Identification and characterization of lactic acid bacteria isolated from traditional cone yoghurt

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Abstract Cone yoghurt is a yoghurt variety produced by adding only pine cones to milk without culture in a limited area of Turkey. The present study was conducted to identify and characterize lactic flora in traditional cone yoghurt. Morphological, cultural, physiological, biochemical, and genotypic characteristics were employed to identify lactic acid bacteria isolates from cones and cone yoghurts. *Streptococcus salivarius* subsp. *thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) were obtained from both cones and yoghurts. Among the isolates, *L. plantarum* was frequently isolated except for these two bacteria (*S. thermophilus* and *L. bulgaricus*). The results indicate that the cone yoghurt has a mixed microflora contrary to the yoghurt produced by the addition of a starter culture and *S. thermophilus*, and *L. bulgaricus* in cone yoghurt originates from the pine cones.

Keywords Cone · Cone yoghurt · Lactic flora · RT-PCR

Introduction

Yogurt is a fermented dairy product produced by specifically inoculation of symbiotic cultures of *Streptococcus salivarius* subsp. *thermophilus* (*S. thermophilus*) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) during fermentation [1]. It is widely consumed in many countries of the world both for its distinctive flavor and for aroma.

Streptococcus thermophilus, which is a bacterium of yoghurt, is an aerobic and facultative anaerobic organism that can be cultivated at 45–52 °C. It grows best between pH 6.0 and 6.5. *L. bulgaricus* is a homofermentative organism that grows best at 45 °C. The optimum pH value (5.2–5.5) for its growth is lower than that for the growth of *S. thermophilus* [1–3].

The main function of these bacteria found in yoghurt, called “yoghurt bacteria,” is to produce L(+) lactic acid by lactose fermentation. When a pure culture is inoculated in milk, *S. thermophilus* breakdown lactose into D(+) lactic acid. The decrease in pH and reduction in oxygen levels provides a suitable environment for the growth of *L. bulgaricus*. In addition, folic acid produced by *S. thermophilus* supports the growth of this bacterium. *L. bulgaricus* quickly converts lactose into D(–) lactic acid [3]. Due to the increased acidity, gradual dissolution of calcium phosphate in casein micelles results in the coagulation of milk. The coagulation begins at pH 5.3 and stops at pH 4.7 [1]. Lactic acid imparts a sour taste to yoghurt. In addition, released metabolites such as acetaldehyde, free aminoacids, acetone, acetoin, diacetyl, dimethyl sulfide, butanone, and low-carbon fatty acids contribute to the flavor and aroma of yoghurt [2].

All strains of yoghurt bacteria cannot provide the desired performance and quality of yoghurt; these

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properties are greatly affected by the type of bacterial strain [4–7]. In Turkey, industrial yoghurts are manufactured using commercial starter cultures obtained from international food culture suppliers. However, such yoghurts do not appeal to the tastebuds of consumers as much as homemade yoghurts.

There are various rumors about how yoghurt was first produced. It is believed that yoghurt was first made by mixing some crushed plant material into milk or adding dew collected on weeds to milk instead of culture. Yoghurt production is still being made by adding cones in a similar method in Simav district in the province of Kütahya in Turkey. A scientific study on the microflora of native plants used in yoghurt production was conducted by Michaylova et al. [8]. They isolated *L. bulgaricus* and *S. thermophilus* from plant samples collected from areas far from human settlements. These isolates were inoculated in milk for obtaining experimental yoghurt. Authors reported that the properties of experimental yoghurt were similar to those of commercial yoghurt. Tavşanlı et al. [9] who studied the proteolytic activity of yoghurt bacteria were able to produce yoghurt using *L. delbrueckii* spp. and *S. thermophilus* strains isolated from plants, rainwater, and dew.

In Turkey, there is a type of yoghurt fermented only by adding pine cones to milk without culture called “cone yoghurt.” Cone yoghurt has been traditionally produced for many years by a limited number of producers in some mountain villages of Kutahya and Eskisehir in Turkey. When preparing this type of yoghurt, preboiled milk is cooled to 42–43 °C. Green cones collected from pine trees in the region are added to the milk, and the mixture is left to ferment for 3–4 h until gel formation. The formed gel is infused into another boiled–cooled milk. This process is repeated three or four times until the desired yoghurt characteristics are attained. No study or publication has previously reported traditional cone yoghurt. This research was performed to identify lactic acid bacteria isolated from cones and cone yoghurt and determine the microorganisms that play a role in yoghurt fermentation.

Materials and methods

Cone yoghurt

In June–December 2015, 12 cone yoghurt samples were purchased from a company engaged in yoghurt manufacturing in Simav district in the province of Kütahya in Turkey. The samples were sent to a laboratory under cold conditions.

Pine cones

During spring, green cones were collected from red pine trees (*Pinus brutia* Ten.) in Simav district in the province of Kütahya. Twenty cones were obtained using sterile gloves and transported to the laboratory in sterile jars under appropriate conditions.

Experimental cone yoghurt production

Filtered fresh raw milk was first heat treated at 85 °C for 30 min and then cooled to 44 °C. According to the method used by local producers, one cone per liter was added to milk. The milk with cones was incubated at 44 °C for approximately 3–4 h until coagulation. The coagulation was retained in a refrigerator for a day and then added into heat-treated–cooled milk in a 5% ratio. At the end of incubation at 44 °C, the acquired coagulation was cooled. The same procedure was repeated twice. In each inoculation, the coagulation produced the previous day was used to inoculate fresh milk. On the final (fourth) day, a more cohesive coagulation similar to traditional yoghurt in terms of texture, flavor, and aroma formed at the end of incubation and was cooled. Cone yoghurts were then stored in a refrigerator for 15 days.

Isolation and identification of lactic acid bacteria from yoghurt and cones

The samples were aseptically weighed, homogenized, and plated on Man–Rogosa–Sharpe agar (MRS, Merck, 110 660), Medium 17 (M17, Merck, 115108), and Lactobacillus Streptococcus Agar (HiMedia M582) differential plates from dilutions prepared using quarter-strength Ringer’s solution Merck, 115525). Plates were incubated under anaerobic conditions at 37 and 45 °C for 24–48 h. Anaerobic conditions were created in airtight jars (Merck, 116387) containing O₂-absorbing and CO₂-releasing sachets (AnaeroGen, Oxoid, AN0025). Anaerobiosis was checked using Anaerotest strips (Merck, 115112). For analysis of the cones, one sample was put into a sterile jar containing 100 mL of sterile 0.9% NaCl solution. The jar was placed for 5 min on a shaker. The rinsing water was used to inoculate on to the abovementioned media. Plates were incubated under the same conditions.

For isolation, selected typical colonies that grew on plates were inoculated onto the same medium using the streak-plating method. After incubation, single bacterial colonies were isolated and subcultured in MRS broth (Merck, 1.10661) and M17 broth (Merck, 1.15029). Isolates were inspected for purity of growth by looking at morphology under a phase-contrast microscope. If any contamination was determined, cultures were restreaked to

ensure purity prior to testing. First, Gram reaction, cell morphology, and catalase activity of pure isolates were determined. Gram-positive, catalase-negative cocci and rods were defined as potential lactic acid bacteria and tested for growth at different temperatures (15 and 45 °C), growth in 4.0 and 6.5% NaCl broth, and gas production from glucose [3, 10]. The selected strains were identified using the API 50 CH test kit (BioMerieux) microtest system [8].

PCR analysis

Streptococcus thermophilus and *L. bulgaricus* isolates were confirmed by PCR. All genomic DNA extraction was performed using DNA-Easy Bacterial DNA Isolation Kit (Bio-Speedy, BS-19-100, Turkey) according to the manufacturer's instructions. The amount of genomic DNA extracted was measured by spectrophotometry (Epoch Microplate Spectrophotometer) and tested for purity and quantity. The extracted DNA was maintained at −20 °C until analysis. Primers and probes used in this study were designed with Primer3 (v.0.4.0) program and synthesized by Bioeksen AR-GE Technologies (Turkey) [11].

The 224 bp long *lacZ* gene (accession EU513599) region was amplified for defining *S. thermophilus* with primer pairs and probe: F 5'-TCGTCCACCTCAAGTTCCTC-3', R 5'-ACGATAAACAGCTACCGCCA-3', 5'-FAM-TCCAAGGGGTTGCTACTTCCA-TAMRA-3'; the 238 bp long *addA* gene (accession AJ252015) region was amplified for defining *L. bulgaricus* with primer pairs and probe: F 5'-CTCATCAACCGGGGCTTTGT-3', R 5'-CAGCTCCCGCATCTCATCTT-3', 5'-FAM-AACAAGAGCGGCGGCTTTGG-TAMRA-3'. All PCR reaction mixtures were prepared using 5 µL 1× PCR buffer (Bioeksen AR-GE Tech.) and 0.5 µM of each primer, 1 U Proofreading Hot-Start Taq DNA Polymerase, 0.5 µM Probe, and 5 ng/µL DNA template for a total 10 µL reaction mixture. *S. thermophilus* ATCC 19258 and *L. bulgaricus* CETC 4005 reference strains were used as a positive control in real-time PCR reactions. Thermocycler (Agilent Stratagene Mx3000P) conditions were as follows: 95 °C for 3 min, 45 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 60 s.

16S rRNA-targeted DNA sequencing

The DNA samples extracted from representative isolates were used by sequencing of the 16S rRNA genes. F: 5'-AGAGTTTGTATCTGGCTCAG-3' and R: 5'-CCATG-CACCACCTGTC-3' primers were used for both *S. thermophilus* (accession NR_118998) and *L. bulgaricus* (accession LC063162). PCR amplification was performed by real-time PCR. Reaction mix was prepared with 5 µL

1× dye mix (including EvaGreen Dye, Bioeksen AR-GE Tech.) and 0.5 µM of each primer, 1 U Proofreading Hot-Start Taq DNA Polymerase and 5 ng/µL DNA template for a total 10 µL reaction mixture. The 16S rRNA gene amplifications were performed under the same temperature as mentioned above. The amplification results were evaluated based on Ct data received from the device in real time. This step was followed by a melting curve analysis from 55 to 95 °C and then cooling to room temperature.

After purification of all 16S rDNA PCR products of *L. bulgaricus* and *S. thermophilus*, PCR amplicons were sequenced by Sanger method using the ABI Prism 377 DNA sequencer (Applied Biosystems, USA). The DNA sequences were compared with the sequences in the GenBank database using internet-based BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). In this study, ≥ 98% sequence similarities were accepted as the cutoffs [12].

Results and discussion

A total of 474 strains from cones, experimental yoghurt, and commercial yoghurt were isolated as potential lactic acid bacteria using the recommended media. According to the results of tests (morphology, Gram staining, growth at different temperatures, growth in different NaCl concentrations, and gas production from glucose), they were classified as *Lactococcus* spp., *Pediococcus* spp., *Leuconostoc* sp., *Enterococcus* sp., *S. thermophilus*, and *Lactobacillus* spp. (Table 1). Some of these isolates were identified using the API 50 CH test kit (Tables 2, 3, 4).

In our study, 160 lactic acid bacteria strains were isolated from pine cones. Fifty-seven strains were identified to potentially be *S. thermophilus*. The 23 randomly selected isolates were identified as *S. thermophilus* (with 90% or greater assurance) based on the API 50 CH test.

The same strains were also confirmed by real-time PCR, which was preferred because it is more rapid and sensitive with a minimized risk of contamination [13]. To identify lactic acid bacteria in dairy products, molecular methods based on bacterial DNA amplification by real-time PCR have been successfully developed for *L. bulgaricus* and *S. thermophilus* [14].

Six selected isolates were further confirmed by applying sequence analysis. Eight of 14 *Lactobacillus* spp. strains from cones were identified as *L. bulgaricus* by the API 50 CH test; this was confirmed by real-time PCR. Three of these were subjected to 16S rRNA sequence analysis and found to be similar (99%) to *L. bulgaricus*. Although these bacteria have also been reported in other plant sources, our study is important from the viewpoint of the detection of *S. thermophilus* and *L. bulgaricus* for the first time in pine

Table 1 Distrubition of isolates from cone yoghurt and cones

Species	Materials			Total
	Cone	Experimental yoghurt	Commercial yoghurt	
<i>Lactococcus</i> spp.	19	38	24	81
<i>Pediococcus</i> spp.	30	29	22	81
<i>Leuconostoc</i> spp.	11	1	–	12
<i>Enterococcus</i> spp.	29	25	29	83
<i>S. thermophilus</i>	57	34	64	155
<i>Lactobacillus</i> spp.	14	34	14	62
Total	160	161	153	474

Table 2 Cone-originated isolates identified with API 50 CH

Strain	n	Identification results	
		Species	Number
<i>Lactococcus</i> spp.	9	<i>La. lactis</i> subsp. <i>lactis</i> 1	7
		<i>La. lactis</i> subsp. <i>lactis</i> 2	2
<i>Leuconostoc</i> spp.	6	<i>Le. mesenteroides</i> subsp. <i>cremoris</i>	6
<i>Streptococcus thermophilus</i>	23	<i>S. salivarius</i> subsp. <i>thermophilus</i>	23
<i>Lactobacillus</i> spp.	14	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	8
		<i>L. plantarum</i> 1	4
		<i>Carnobacterium maltaromaticum</i>	1
		<i>L. fructivorans</i>	1

Table 3 Commercial yoghurt-originated isolates identified with API 50 CH

Strain	n	Identification results	
		Species	Number
<i>Lactococcus</i> spp.	7	<i>La. lactis</i> subsp. <i>lactis</i> 1	6
		<i>La. lactis</i> subsp. <i>lactis</i> 2	1
<i>Leuconostoc</i> spp.	1	<i>Le. mesenteroides</i> subsp. <i>cremoris</i>	1
<i>Streptococcus thermophilus</i>	7	<i>S. salivarius</i> subsp. <i>thermophilus</i>	7
<i>Lactobacillus</i> spp.	28	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	25
		<i>L. plantarum</i> 1	3

Table 4 Experimental yoghurt-originated isolates identified with API 50 CH

Strain	n	Identification results	
		Species	Number
<i>Lactococcus</i> spp.	6	<i>La. lactis</i> subsp. <i>lactis</i> 1	6
<i>Streptococcus thermophilus</i>	45	<i>S. salivarius</i> subsp. <i>thermophilus</i>	45
<i>Lactobacillus</i> spp.	9	<i>L. delbrueckii</i> subsp. <i>bulgaricus</i>	5
		<i>L. plantarum</i> 1	4

cones. This determination implies that bacteria in cone yoghurt originate from pine cones. Some authors have also reported that these two bacteria can be found in natural sources. A similar study on yoghurt production with plant material was conducted by Michaylova et al. [8]. They studied the isolation of *L. bulgaricus* and *S. thermophilus* from plant samples (*Calendula officinalis*, *Capsella bursa-pastoris*, *Chrysanthemum*, *Cichorium intybus*,

Colchicum, *Cornus mas*, *Dianthus*, *Galanthus nivalis*, *Hedera*, *Nerium oleander*, *Plantago lanceolata*, *Prunus spinosa*, *Rosa*, *Tropaeolum*, etc.) collected from four geographical regions known for their high-quality yoghurt in Bulgaria. Plant samples were obtained from several sites that were away from human settlements to avoid potential contamination from homemade or commercial yoghurt. Bacterial growth was detected in 319 of 665 samples, and

202 strains containing 70 rod-shaped bacteria and 132 coccial bacteria were isolated from positive samples. In the mentioned study, most of the rods (63 of 70) were classified as *L. bulgaricus*, and the remaining seven rods were classified as *L. helveticus*. Eight and 124 of the coccial bacteria were identified as *La. lactis* and *S. thermophilus*, respectively. Tavsanlı et al. [8] isolated *L. delbrueckii* subsp. and *S. thermophilus* strains from some plants, rain-water, and dewdrops, and used them in another study on the bacteriolytic activities of lactic acid bacteria.

Three hundred and fourteen strains were isolated from commercial cone yoghurt and experimental cone yoghurt samples in the present study. Ninety eight of the isolates were classified as *S. thermophilus* and 48 as *Lactobacillus* strains. Fifty-two representative *S. thermophilus* isolates (7 from experimental yoghurt and 45 from commercial yoghurt) were tested with API 50 CH and identified as *S. thermophilus*. Thirty-seven representative *Lactobacillus* were tested with API 50 CH, and 30 of them (5 from experimental yoghurt and 25 from commercial yoghurt) were identified as *L. bulgaricus*.

Seventy-four of 75 isolates identified as *S. thermophilus* and all the 38 isolates identified as *L. bulgaricus* strains by the API 50 CH test were also confirmed by PCR analysis.

Nucleotide base sequences of *S. thermophilus* and *L. bulgaricus* 16s ribosomal DNA (rDNA) provide an accurate basis for identification [15]. Twenty *S. thermophilus* (6 from cones, 8 from experimental yoghurt, and 6 from commercial yoghurt) and 11 *L. bulgaricus* (3 from cones, 5 from experimental yoghurt, and 3 from commercial yoghurt) isolates confirmed by PCR were also analyzed by 16s rRNA sequencing and compared to sequences in the GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Seventeen *S. thermophilus* and 8 *L. bulgaricus* strains showed similarity rates of 98–100% (Table 5).

According to the research conducted by Drancourt et al. [12], a 16s rDNA sequence similarity of 99% with the prototype strain sequence in GenBank was defined as identification to the species level; 16s rDNA sequence similarity of 97% with the prototype strain sequence in GenBank was defined as identification to the genus level. Janda and Abbott [16], in their recommended guidelines, suggested that a minimum of > 99%, and ideally > 99.5%, sequence similarity be used as the criteria for species identification.

The results of the study showed that yoghurt bacteria (*S. thermophilus* and *L. bulgaricus*) were present in pine cones and cone yoghurt microflora. The yoghurt bacteria cannot survive after heat treatment during yoghurt production. Therefore, the presence of these bacteria in yoghurt without starter culture refers to a subsequent contamination. In this study, isolation of yoghurt bacteria from cones and

experimental yoghurt indicated that the origin of these bacteria is cones.

There is no study on microbiology of cone yoghurts produced without culture. However, a lot of study has been conducted in Turkey on artisanal and industrial yoghurt produced using culture. Aslim et al. [17] reported that the 20 strains of *Streptococcus* and 22 strains of *Lactobacillus* isolated from homemade and industrial yoghurt were identified as *S. thermophilus* and *L. bulgaricus*, respectively, from village yoghurt and commercial yoghurt. Erkus [18], who studied the characterization of yoghurt starter, isolated 66 cocci and 71 bacilli from artisanal yoghurt and reported that all the bacilli isolates were found to be *L. bulgaricus*, but cocci isolates showed highly variable sugar fermentation results and only seven of them were characterized as *S. thermophilus* according to biochemical identification. Aslim and Beyatli [19] determined that 73 of 81 strains of *Lactobacillus* isolated from the yoghurt samples collected from different regions of Turkey were defined as *L. bulgaricus*.

In our study, the results of isolation and identification showed that cone yoghurt has a complicated microflora. It was also determined other lactic acid bacteria as well as yoghurt bacteria (*S. thermophilus* and *L. bulgaricus*) and non-lactic acid bacteria are present in cone yoghurt. Isolates from the recommended media for lactic acid bacteria were classified as *Lactococcus* spp. (62 strains), *Pediococcus* spp. (51 strains), *Enterococcus* spp. (54 strains), and *Leuconostoc* spp. (1 strain). Some of these isolates were tested by API 50 CH. Thirteen representative *Lactococcus* isolates were identified as *La. lactis* subsp. *lactis*. One *Leuconostoc* isolate was identified as *Le. mesenteroides* subsp. *cremoris*. The same bacteria were also determined in cones. A total of 19 *Lactococcus*, 30 *Pediococcus*, and 11 *Leuconostoc* were isolated from pine cones. Nine representative *Lactococcus* isolates were identified as *La. lactis* subsp. *lactis* and six *Leuconostoc* isolates were identified as *Le. mesenteroides* subsp. *cremoris*. These findings provide information about the origin of lactic acid bacteria in yoghurt.

Enterococci are a part of the normal intestinal flora and frequently used as fecal indicator bacteria. If there is a contamination to milk during milking and processing, these bacteria can lead to various problems in fermented milk products because they are tolerant of a range of pH values. *Enterococcus faecalis*, *E. faecium*, and *E. bovis* are the main species isolated from contaminated fermented milk [20]. Although the presence of enterococci was associated with fecal contamination in the past, it is known that these bacteria may be available in environmental samples such as soil, plants, and insects. In the present study, 83 *Enterococcus* spp. (19 from cones, 25 from commercial yoghurt, and 29 from experimental yoghurt) were isolated from the

Table 5 16s rRNA Gene sequencing similarity scores of selected isolates

Strain	16s sequence analyses results (% similarity)	Accession number	Origin
<i>S. thermophilus</i>	99	gb HQ721249.1	Commercial yoghurt
<i>S. thermophilus</i>	99	gb EU419603.1	
<i>S. thermophilus</i>	99	gb EU419603.1	
<i>L. bulgaricus</i>	99	gb KJ939317.1	
<i>L. bulgaricus</i>	99	gb KJ868757.1	
<i>L. bulgaricus</i>	99	gb CP013610.1	
<i>L. bulgaricus</i>	99	gb HQ293103.1	
<i>L. bulgaricus</i>	99	gb KJ026657.1	Experimental yoghurt
<i>S. thermophilus</i>	99	ref NR_074827.1	
<i>S. thermophilus</i>	99	ref NR_074827.1	
<i>S. thermophilus</i>	99	ref NR_074827.1	
<i>S. thermophilus</i>	99	gb EU419603.1	
<i>S. thermophilus</i>	99	ref NR_074827.1	
<i>S. thermophilus</i>	100	gb KJ890358.1	
<i>S. thermophilus</i>	99	ref NR_074827.1	
<i>S. thermophilus</i>	100	gb KJ890358.1	
<i>S. thermophilus</i>	98	gb EU419603.1	
<i>S. thermophilus</i>	99	gb KJ890358.1	Pine cone
<i>S. thermophilus</i>	99	dbj LC_096241.1	
<i>S. thermophilus</i>	99	gb EU419603.1	
<i>S. thermophilus</i>	99	gb EU419603.1	
<i>S. thermophilus</i>	99	gb HQ721249.1	
<i>L. bulgaricus</i>	99	gb KJ026576.1	
<i>L. bulgaricus</i>	99	gb HQ293060.1	
<i>L. bulgaricus</i>	99	gb HQ293060.1	

medium for lactic acid bacteria. A selected isolate (from pine cone) was identified by sequence analysis. The 16s rRNA gene sequences showed 99% similarity with *E. faecium* in the database. An isolate identified as *S. thermophilus* by API 50 CH showed 99% 16s rRNA sequence similarity with *E. durans*. There are studies reporting that enterococci were present in classic yoghurt and could live for a long time [21, 22].

Lactobacillus plantarum is a short rod-shaped, gram-positive lactic acid bacterium. It can grow at temperatures between 15 and 45 °C and under acidic conditions with a pH as low as 3.2. *L. plantarum* is a facultative heterofermentative microorganism that ferments sugars to produce lactic acid, ethanol, or acetic acid [23]. It is commonly found in the intestines of humans and mammals. It is also present in many foodstuffs such as dairy products, vegetables (pickles, sauerkraut, sourdough, etc.) and meat sausages [24]. *L. plantarum* is considered as a safe probiotic because it helps to limit the colonization of the pathogenic bacteria in the intestine and has other beneficial effects on human health [25–28]. In this study, four of 14 *Lactobacillus* spp. isolated from cones, three of 28

Lactobacillus spp. isolated from commercial yoghurt, and three of nine *Lactobacillus* spp. isolated from experimental yoghurt were identified as *L. plantarum* by the API 50 CH test. These results indicate that *L. plantarum* can play an important role in cone yoghurt and originates from the pine cones used in fermentation of milk. *L. plantarum* is capable of producing exopolysaccharides [27]. Exopolysaccharide-producing bacteria were used to improve the rheological properties, texture, and mouthfeel in fermented dairy products such as cheese and yoghurt [29].

Two of 14 rod-shaped isolates (from the pine cone samples) were identified as *Carnobacterium maltaromaticum* and *L. fructivorans* by the API 50 CH test. *Carnobacterium* is a gram-positive lactic acid bacterium and a member of the *Leuconostocaceae* family. *C. maltaromaticum* is frequently isolated from the natural environment. This bacterium is able to grow at low temperatures, anaerobically, and under increased CO₂ concentrations [30]. This opportunistic lactic acid bacterium is present in a low proportion in raw milk. It can grow in milk without coagulation because of a low production of L(+)-lactic acid from lactose due to a weak β-

galactosidase activity with no competition from starter lactic acid bacteria. These species synthesize different flavoring compounds such as 3-methylbutanal [31]. Cailiez-Grimal et al. [32] reported that this bacterium was determined in various French soft-flavored or washed rind cheeses. *C. maltaromaticum* has attracted the attention of researchers because it can inhibit the growth of foodborne pathogens such as *Listeria monocytogenes* due to its ability to produce bacteriocins [33]. *L. fructivorans* obtained isolate from cone is known to be detrimental to products for acidic food like mayonnaise, salad dressings, vinegar preserves, sake [34, 35].

According to the results of this study, *S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are available in cone yoghurt produced without culture addition. The number of lactobacilli is significantly lower than that of streptococci. Cone yoghurt has a rich and mixed microflora. In addition to yoghurt bacteria, other lactic acid and non-lactic acid bacteria are found in cone yoghurt. These bacteria also likely contribute to the development of the flavor and the formation of the cone yoghurt. Because *L. plantarum* comprises a significant proportion of the isolates, it may play an important role in yoghurt formation. Lactic acid bacteria in cone yoghurt originate from pine cones because the same bacteria are found in cones. These isolates, particularly yoghurt bacteria, should be evaluated in terms of their potential to be used as commercial yoghurt starter cultures by performing necessary tests and analyses.

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