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Isolation of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* from nature: Technological characterisation and antibiotic resistance

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Abstract: Yoghurt fermenting bacteria were isolated from natural sources including plants, dew, and rain samples (total of 300 samples) by the same methods nomadic peoples used for several centuries in Turkey. Inoculation into the reconstituted skim milk followed by planting on specific media and matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis allowed for the identification of 18 *Lactobacillus delbrueckii* subsp. and 26 *Streptococcus thermophilus*. A multiplex polymerase chain reaction (PCR) assay applied to lactobacilli enabled the identification of 5 isolates as *L. delbrueckii* subsp. *bulgaricus*. The isolates showed a varying range of acidification rates and proteolytic activity in reconstituted skimmed milk (RSM). *S. thermophilus* isolates showed a broader range of resistance and the most frequent resistance was observed for streptomycin (69.2%), gentamycin (65.3%), clindamycin (61.5%), ampicillin (61.5%), kanamycin (53.8%), and erythromycin (50%). For *L. delbrueckii* subsp. the highest resistance was determined for vancomycin (38.8%), ciprofloxacin (33.3%), and penicillin (27.8%). The frequency of multiple resistance was tested on 14 different antimicrobials determining that 19 *S. thermophilus* (73%) and 3 *L. delbrueckii* subsp. (16.7%) demonstrated resistance to more than three different antibiotics. In contrast to this wide-ranging resistance, five isolates from each genus were found to be susceptible to all tested antibiotics. The present study indicates that lactic acid bacteria (LAB) isolated from nature may have broad-range of resistance to antibiotics and could be a source for the transfer of resistance.

Keywords: yoghurt bacteria; natural isolates; characterisation; antibiotic resistance

In Anatolia, villagers and nomadic peoples used to produce yoghurt from microbial sources such as plants, dew, and rainwater. It is thought nature, in particular plants, is the natural habitat of yoghurt fermenting bacteria. Thus, it has been hypothesised that these bacteria have adapted to milk environments and have been used in the manufacturing of yoghurt for a long time. In recent decades, the strains *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* have been isolated from plants and cones samples

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collected from natural environments (Michaylova et al. 2007; Bostan et al. 2017). The technological properties and enzymatic content of these isolates were confirmed to be suitable for milk fermentation and could be used as yoghurt starters.

Lactic acid bacteria (LAB) are involved in the fermentation process of various dairy products such as yoghurt, cheese, cultured butter, and sour cream as well as some other foodstuffs including sausages, pickles, olives, and sauerkraut. As the most important microorganism used for dairy fermentations, LAB are responsible for the formation of flavour and texture by producing large amounts of lactic acid together with other metabolites (Crowley et al. 2013). In yoghurt manufacturing, fermentation of lactose by the symbiotic growth of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* results in a decrease of pH through glycolysis which leads to the formation of organic acids such as lactic acid and relatively lower amounts of other acids such as acetic and, propionic acids in the final product (Erkus et al. 2014). A decrease in pH correspondingly contributes to the clotting of caseins resulting in the characteristic texture and taste of yoghurt (Driessen et al. 1982). Both of these microorganisms are thermophilic, and the temperature for their optimal symbiotic growth is between 42 °C to 45 °C. During their symbiotic growth, *L. delbrueckii* subsp. *bulgaricus* has relatively higher proteolytic activity, hydrolysing milk casein to produce oligopeptides and free amino acids which have a stimulatory effect on the growth of *S. thermophilus*. In turn, decreased pH, reduced oxygen, increased carbon dioxide, and formic acid produced by *S. thermophilus* stimulate the growth of *L. delbrueckii* subsp. *bulgaricus* (Driessen et al. 1982; Bostan et al. 2017).

LAB known for their beneficial effect on human health may also be a reservoir for antibiotic resistance and for dissemination of resistance genes to other pathogenic bacteria (Morandi et al. 2015). LAB isolated from different fermented products including dairy products, meat, and plants were shown to display resistance profiles as well as resistance genes. Antibiotic resistance which may be acquired by horizontal gene transfer such as conjugative plasmids or transposons in LAB may lead to the dissemination of these genetic traits to other bacteria. It has been recently stated that LAB used in the food industry may have potential in the dissemination of these resistance traits through the food chain (Mathur and Singh 2005).

The present study was conducted to provide evidence on the presence of LAB from different natural ecosystems. Some technological characteristics as well antibiotic resistance profiles of isolates were studied. Obtained data could provide the evidence not only on the selection of suitable bacteria as starter cultures in dairy fermentation, but also on the potential of food fermenting bacteria in the dissemination of resistance.

MATERIAL AND METHODS

Sample collection. A total of 300 plants, rainwater and dewdrop, samples (100 samples from each) were collected in springtime (March, April and first weeks of May) from rural areas outside of a 50 km radius from industrial production, and settlements in 10 different locations in Marmara and Aegean Regions in Turkey. Plant samples were chosen from amongst *Nasturtium officinale*, *Rumex acetosella*, *Triticum*, *Hordeum vulgare*, *Lolium*, *Sinaps arvensis*, *Cupressocyparis*, and *Robenis pseudocacia* species. Approximately 150 g of fresh plant samples with lengths of 15 cm and composed of both leaf and stem parts were gently collected and replaced in a sterile laboratory bag (Isolab, Germany) before transferring to the laboratory. The dew samples were collected using traditional methods preferred by villagers. Briefly, at sunrise, a sterilised muslin cloth (15 × 20 cm size) was used for swabbing over the plants. The process is stopped when the muslin is saturated. Finally, the muslin cloths were transferred to sterile plastic bags. For the rain samples, sterile jars were left open on rainy days in pastoral areas until they became filled with 50 mL to 100 mL of rainwater.

Milk clotting, bacterial isolation, and identification. The plant (P; approximately 150 g), rainwater (RW), and dewdrop (DD; min 50 mL for each) samples were incubated (incubator IN55; Memmert, Germany) in 1 L of reconstituted skimmed milk (RSM) at 42–44 °C for 72 h. Samples in which coagulation was observed were then appropriately diluted and inoculated to Man Rogosa Sharpe (MRS) agar (Oxoid, CM1153) plates for lactobacilli and M17 agar (Oxoid, CM0785) plates for streptococci. The plates were then incubated at 42–44 °C for 72 h in anaerobic (MRS) or aerobic (M17) conditions. Colonies representing the LAB profile for their morphology (Gram staining), catalase production, and fermentation of carbohydrates (Sherman 1937), were stored at –20 °C for further analyses (MALDI-TOF MS; Bruker Daltonics, Germany).

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) identification. The identification of the isolates by matrix-assisted laser desorption/ionization-time-of-flight

https://doi.org/10.17221/296/2020-CJFS

mass spectrometry (MALDI-TOF MS) was performed on an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics GmbH, Germany) for the automatic acquisition of the mass spectra in the linear positive mode within a range of 4 000–10 000 kDa, according to the instructions of the manufacturer. The instrument was periodically calibrated by using the Bruker Daltonics bacterial test standard, according to the instructions of the manufacturer. Distilled water was used as a negative control.

For the identification of bacteria, the surface of the investigated colony was touched with a sterile pipette tip and transferred directly to the target part of the mass spectrometer. The deposited bacteria were overlaid with 1 µL of HCCA matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and air-dried at room temperature to allow co-crystallisation with the experimental sample. The spectra were then acquired and compared by using the BioTyper software 3.1 (Bruker Daltonics, Germany).

Differentiation of *L. delbrueckii* subsp. *bulgaricus* by Multiplex polymerase chain reaction (PCR). Subspecies level identification of *L. delbrueckii* was performed by polymerase chain reaction (PCR) method using primers specific to *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*. For this purpose, the Chelex 100 (Sigma, USA) resin-based technique was used for DNA extraction. Fresh culture of each isolate (1 mL) was centrifuged (1-14 K; Sigma, USA) at 12 000 g for 3 min. Chelex 100 resin (6%, 200 µL) and proteinase K (2 µL) were added to each pellet. The suspensions were mixed and incubated at 55 °C for 40 min and then at 100 °C for 8 min in a shaking block heater (DB-4S; BioSan, Latvia). Thereafter, the mix was centrifuged at 12 000 g for 3 min and the supernatant containing template DNA was stored at –20 °C (Freezer 2080 MultiMode; Arçelik, Turkey). The PCR was performed according to Cremonesi et al. (2011) by using primers targeting the lacZ 395 bp size gene (5'-GGAAGACTCCGTTTTGGTCA-3') (5'-AGTTCAAGTCTGCCCCATTG-3') for *L. delbrueckii* subsp. *bulgaricus* and ddpE 217 bp size gene (5'-TGCCAAGCTCTACTCCGTTT-3') (5'-GTCAAGCGGCATAGTGTCAA-3') for *L. delbrueckii* subsp. *lactis*. Briefly, the PCR procedure was applied in a total volume of 50 µL including 20 µM of each primer, ~30 ng of the template DNA, 2 units (U) of Taq DNA polymerase, 1.5 mM of $MgCl_2$, 5 µL of 10X PCR buffer, 200 µM each of dNTPs and nuclease-free water. DNA amplification was carried out in a thermal cycler (SCM 96G; Runik, Turkey) with an initial denaturation of DNA at 94 °C for 15 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 68 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were visualised by 2% agarose gel electrophoresis, and stained with ethidium bromide.

Evaluation of proteolysis and acidification. The proteolysis was determined using a fast-slow differential agar (FSDA) medium (Cibik et al. 2010). The media was composed of agar agar (6 g), sodium glycerophosphate (9.5 g), bacto litmus (0.5 g), and skim milk powder (50 g). The bacterial suspension was spread onto the media and incubated at 42 °C for 48 h (incubator IN55; Memmert, Germany). Large opaque colonies which appeared yellowish-white in colour, and were surrounded by a pink halo on FSDA plates were regarded as proteolysis positive (Prt^+), whereas proteolysis negative (Prt^-) strains formed small, white, translucent colonies without the halo. The proteolytic capacity of the strains was evaluated as weak (0.2–0.5 mm), moderate (0.6–0.9 mm), or strong (1–3 mm) by measuring the diameter of the halo around the colony (Caliper; Temak, Turkey).

The acidification was performed according to Cogan et al. (1997) in RSM. Ten millilitres of RSM were inoculated with 1% late exponential growth phase culture grown in MRS or M17 broth and incubated at 44 °C. The change in pH was followed over 24 h using a pH meter (S220-K; Mettler Toledo, USA).

Proteolysis and acidification tests were repeated triplicate and representative results are presented.

Antibiotic susceptibility testing. The susceptibility of isolates to antibiotics was assessed by the disk diffusion method according to recommendations from the EFSA (2012). For this purpose, quinupristin/dalfopristin (15 µg), ampicillin (10 µg), vancomycin (30 µg), linezolid (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), streptomycin (10 µg) tetracycline (30 µg), clindamycin (2 µg) erythromycin (15 µg) cefotaxime (30 µg) kanamycin (30 µg), chloramphenicol (30 µg), and penicillin [10 international units (IU)] antibiotic discs were studied. *S. pneumonia* ATCC 49619 was used as a quality control microorganism. The minimum inhibitory concentrations (MICs) of lactobacilli were evaluated using the E-test method according to recommendations from the EFSA (2012). For this purpose, the MICs of ampicillin, amoxicillin-clavulanic acid, vancomycin, clindamycin, and chloramphenicol antibiotics were determined, and interpreted according to the guidelines. Considered MICs values for ampicillin, amoxicillin-clavulanic acid, and chloramphenicol were > 8 µg mL^{-1}, for clindamycin > 4 µg mL^{-1}, and for vancomycin > 2 µg mL^{-1}.

https://doi.org/10.17221/296/2020-CJFS

RESULTS AND DISCUSSION

Sample collection. In Anatolia (Turkey), for several centuries plants, cones, dew, and rainwater, have been used for milk clotting in order to produce yoghurt at home. These were collected in the spring months of March to May when vegetation and plant growth is optimal. No milk clotting would be observed if a collection was made in summer or fall months which may be linked to relatively lower humidity and decreased vegetation in Turkey during these dry months. This would also be the reason why villagers prefer this period of the year to collects samples.

Bacterial isolation and identification. Upon incubation for 72 h in sterilised RSM at 42–44 °C, milk clotting was observed for 55 samples (17 P, 20 RW, and 18 DD). No clotting was observed for the remainder of the samples despite extended incubations. Appropriate dilutions from these clotted samples allowed for isolation of 46 colonies on MRS and 50 colonies on M17 for their morphological characteristics and basic biochemical tests. MALDI-TOF MS identification of these isolates is summarised in Table 1. The majority of isolates from MRS were identified as *L. delbrueckii* subsp., and *L. plantarum*. The other identified species were *L. fermentum*, *L. paraplantarum*, *L. casei*, and *L. acidophilus*. *L. delbrueckii* subsp. (18 isolates) that were not identified at sub-species level were then PCR tested, and five were identified as *L. delbrueckii* subsp. *bulgaricus*. Amongst the streptococci, *S. thermophilus* was the predominant species (26 isolates). The source of bacteria in DD, and RW might be a result of contamination from the surroundings such as plants or soil. LAB including *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Michaylova et al. 2007; Bostan et al. 2017), *L. coryniformis* (Weinrichter et al. 2001), *L. helveticus* (Dimitrov et al. 2005), and *L. plantarum* (Salvucci et al. 2016) have been isolated from plants from different parts of the world.

Table 1. Isolation of lactic acid bacteria (LAB) from different natural sources and identification by MALDI-TOF MS

Bacteria	Isolates			
	P	RW	DD	total
Lactobacillus delbrueckii[a]	3	8	7	18[a]
Lactobacillus plantarum	14	2	3	19
Lactobacillus paraplantarum	–	1	–	1
Lactobacillus fermentum	–	3	3	6
Lactobacillus casei	–	–	1	1
Lactobacillus acidophilus	–	1	–	1
Streptococcus thermophilus	4	10	12	26
Enterococcus faecium	11	3	4	18
Enterococcus faecalis	1	3	1	5
Streptococcus uberis	–	1	–	1

[a]Five isolates were identified by polymerase chain reaction (PCR) as *Lactobacillus delbrueckii* subsp. *bulgaricus*; P – plant; RW – rainwater; DD – dew drop

Evaluation of proteolysis and acidification. The acid production ability of isolates was calculated by measuring the pH change over time in RSM (Figure 1). The mean pH value measured for *S. thermophilus* isolates was 4.26 at the end of a 24 h incubation and 3.95 for *L. delbrueckii* subsp. *bulgaricus* isolates.

The proteolytic activity of isolates was determined on differentiation agar as weak, moderate, or strong activity and the results are presented in Table 2. Isolates showed the strain dependent distribution for proteolysis and stronger activity was observed for six *S. thermophilus*, and one *L. delbrueckii* subsp. *bulgaricus*. Proteolytic enzymes of LAB play an important role in dairy since the resulting degradation products are the precursors for aromatic compounds in the final product. As seen in Table 2, six (23%) *S. thermophilus* and one *L. delbrueckii* subsp. *bulgaricus* exhibited strong proteolysis on the FSDA medium. Strong proteolysis was previously reported for *S. thermophilus* isolated from artisanal yoghurts in Turkey (Erkus et al. 2014) and natural isolates from Bulgaria (Urshev et al. 2014). Likewise, Michaylova et al. (2007) reported higher proteolytic activity for their *L. delbrueckii* subsp. *bulgaricus* isolates.

In yoghurt production, acidification is the main stage for milk clotting and product formation. Glycolysis and lactic acid production of LAB is a complex and strain-dependent phenomenon requiring the involvement

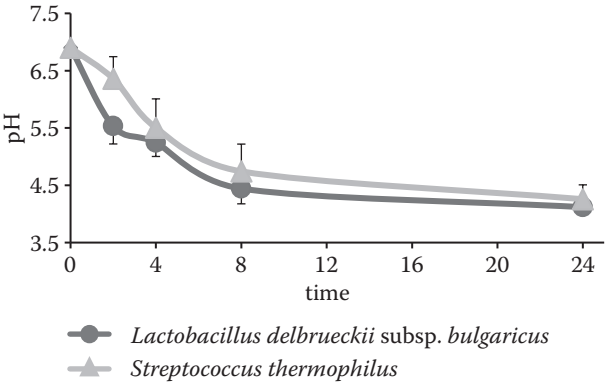

Figure 1. Acidification of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in reconstituted skimmed milk (RSM)

The mean values of all isolates were presented

https://doi.org/10.17221/296/2020-CJFS

Table 2. Evaluation of proteolysis on fast-slow differential agar (FSDA)

Bacteria	Weak	Moderate	Strong
Streptococcus thermophilus	S24, S35, S36, S59, S63, S67, S68, S69, S70, S71, S74, S111, S116	S60, S61, S75, S81, S83, S113, S114	S40, S62, S69, S81, S83, S112,
Lactobacillus delbrueckii subsp. *bulgaricus*	L28, L112, L114	L113	L58

of several enzymes, thus indicating variability among the strains. The rate of acidification is an important parameter in yoghurt production. Selections of strains having weak post acidification properties may be an effective perspective to reduce post acidification of yoghurt during the shelf-life period (Xu et al. 2015). In the present study, the mean pH values were 6.35; 4.26 for *S. thermophilus*, and 5.59; 3.95 for *L. delbrueckii* subsp. *bulgaricus* at 2nd and 24th h, respectively, suggesting relatively higher acidifying activity for lactobacilli.

Antibiotic susceptibility testing. Data related to antibiotic resistance of isolates are shown in Table 3. Most of the lactobacilli showed resistance to at least one antibiotic. Clindamycin, and ciprofloxacin resistance were common among *L. delbrueckii* subsp. *bulgaricus* isolates (60%); however, other *L. delbrueckii* subsp. had higher resistance to vancomycin (38.8%). Interestingly, any tested isolates were resistant to quinupristin/dalfopristin, tetracycline, and chloramphenicol. *S. thermophilus* had a much greater resistance profile. Resistance was seen towards streptomycin (69.2%), followed by clindamycin (61.5%), ampicillin (61.5%), kanamycin (53.8%), erythromycin (50%), ciprofloxacin (46.1%), tetracycline (34.6%), linezolid (30.7%) and chloramphenicol (23%). Only one isolate exhibited slight resistance to vancomycin.

In the present study, the corresponding aim was to check the resistance of natural isolates of LAB/yoghurt bacteria to different antimicrobials through the use of a disk diffusion test. Remarkably, the multidrug resistance (MDR) profile was relatively common among *S. thermophilus*. Of the 26 tested isolates, 19 had resistance to at least three different antibiotics (Table 4). Isolate S69 was the most striking sample with resistance to 11 antibiotics while isolates S24, S36, S59, and S71 were each resistant to 10 antibiotics.

Antibiotic resistance is an undesirable characteristic for LAB since these bacteria are utilised as starters in the fermentation process of various foodstuffs as well some being used as probiotics. Some can survive in the human intestinal system, and interact with the indigenous bacteria to transfer genes responsible for the resistance. The present study evidenced the resistance of some *L. delbrueckii* subsp. to vancomycin, clindamycin am-

Table 3. Antibiotic resistance of lactobacilli and *Streptococcus thermophilus*

Antibiotics	*Lactobacillus delbrueckii* subsp.	*Lactobacillus delbrueckii* subsp. *bulgaricus*	*Streptococcus thermophilus*
	number and % of resistant isolates		
Quinupristin/dalfopristin	0	0	3 (11.5%)
Ampicillin	2 (11.1%)	0	16 (61.5%)
Vancomycin	7 (37.8%)	0	1 (3.8%)*
Linezolid	1 (5.5%)	0	8 (30.7%)
Gentamicin	2 (11.1%)	0	17 (65.3%)
Ciprofloxacin	6 (33.3%)	3 (60%)	12 (46.1%)
Streptomycin	3 (16.6%)	0	18 (69.2%)
Tetracycline	0	0	9 (34.6%)
Clindamycin	3 (33.3%)	3 (60%)[a]	16 (61.5%)
Erythromycin	2 (11.1%)	1 (20%)[a]	13 (50.0%)
Cefotaxime	1 (5.5%)	0	9 (34.1%)
Kanamycin	1 (5.5%)	0	14 (53.8%)
Chloramphenicol	0	1 (20%)[a]	6 (23.0%)
Penicillin	5 (27.8%)	0	6 (23.0%)

Analysed by the disk diffusion test; [a]confirmed by E-test; *slight inhibition

https://doi.org/10.17221/296/2020-CJFS

Table 4. Multidrug resistance (MDR) of *Lactobacillus delbrueckii* subsp. and *Streptococcus thermophilus* analysed by the disk diffusion method

Bacteria	Isolates	MDR observed antibiotics counts
L. delbrueckii subsp.	L68	7
	L75	5
	L111	3
S. thermophilus	S69	11
	S24, S36, S59, S71	10
	S68, S70	9
	S35, S61, S83	8
	S63, S75	7
	S67, S82	6
	S78, S80, S114, S116	5
	S40	4

picillin, and chloramphenicol antibiotics. Vancomycin resistance in lactobacilli is due to the presence of a D-lactate in the peptide chain in their cell wall (Klein et al. 2000; Wang et al. 2019). Although vancomycin resistance is widespread among *L. delbrueckii* subsp. it is not a common feature for all lactobacilli. In the literature, inconsistent reports have been published regarding the antibiotic resistance of *L. delbrueckii* subsp. *bulgaricus*. Most reports highlighted resistance to ampicillin (Karapetkov et al. 2011; Zhou et al. 2012), amoxicillin-clavulanic acid (Tang et al. 2007), chloramphenicol (D'Aimmo et al. 2007; Zhou et al. 2012), clindamycin (Karapetkov et al. 2011), streptomycin (Zhou et al. 2012; Zhang et al. 2013), kanamycin (Nawaz et al. 2011; Zhou et al. 2012), gentamycin (Nawaz et al. 2011; Zhou et al. 2012), ciprofloxacin (Nawaz et al. 2011), and bacitracin (Akpinar et al. 2011), while some reported susceptibility to clindamycin (D'Aimmo et al. 2007; Nawaz et al. 2011; Georgieva et al. 2015), chloramphenicol (Georgieva et al. 2015), tetracycline (Zhang et al. 2013; Georgieva et al. 2015), erythromycin (Zhang et al. 2013), ampicillin (Nawaz et al. 2011; Zhang et al. 2013; Georgieva et al. 2015), and bacitracin (D'Aimmo et al. 2007; Akpinar et al. 2011). This discrepancy may be explained by the origin of the isolates, methods applied in studies, and/or culture conditions such as media used for culturing.

S. thermophilus isolates exhibited similar or even lower resistance profiles. Most of the isolates were resistant to various antibiotics except for vancomycin. As previously reported, resistance to vancomycin is not a general feature for *S. thermophilus* contrary to enterococci (Nawaz et al. 2011; Zhang et al. 2013; Georgieva et al. 2015). Analysis of the resistance/susceptibility profile of this bacterium evidenced that it could carry resistance to erythromycin (Karapetkov et al. 2011; Nawaz et al. 2011; Zhang et al. 2013), clindamycin (D'Aimmo et al. 2007; Karapetkov et al. 2011; Zhang et al. 2013), tetracycline (Tang et al. 2007; Nawaz et al. 2011; Morandi et al. 2015) and chloramphenicol (Tang et al. 2007; Nawaz et al. 2011; Zhou et al. 2012). Results obtained from the present study suggest that even if an isolation location is far removed from human settlement, bacteria may carry one or more antibiotic resistance profiles.

MDR has gained importance in recent decades. Starter bacteria used in the food industry may carry resistance traits of several antibiotics. In the present study, the majority of the *S. thermophilus* strains were resistant to more than three antibiotics. Interestingly, one isolate showed resistance to 11 different antibiotics.

CONCLUSION

More detailed studies are necessary to gain a deeper understanding of the use of bacteria in dairy technology in terms of traditional starter cultures. More attention should be given to LAB or other fermentation bacteria for their potential role in the transfer of resistance materials among microorganisms.

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