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Full Length Research Paper

Antimicrobial activity and antibiotic resistance of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains isolated from Turkish homemade yoghurts

Asli Akpinar, Oktay Yerlikaya* and Sevda Kiliç

Department of Dairy Technology, Faculty of Agriculture, Ege University, 35100, Bornova – zmir, Turkey.

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The aim of this study was to determine the inhibitive effect of 25 *Lactobacillus delbruecki* ssp. *bulgaricus* and 16 *Streptococcus thermophilus* strains isolated from 30 different homemade yoghurts on several pathogen and contaminant bacteria. The antibiotic resistance of these bacteria was also determined. All of *Lactobacillus bulgaricus* strains exhibited antimicrobial activity against *Escherichia coli*, whereas all of *S. thermophilus* strains exhibited the same activity against *Klebsiella pneumoniae*. None of *L. bulgaricus* strains were resistant to the polymixin-B, only the OL4 strain has shown resistance to bacitracin. While some strains of *S. thermophilus* like C6 and SL4 exhibited resistance to novobiocin, SY72, M3, C1M, and F1M were shown to optochin. ET6 and SY73 strains were found to be resistant in both novobiocin and optochin.

Key words: Antibiotic resistance, antimicrobial activity, homemade yoghurt, lactic acid bacteria, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus.*

INTRODUCTION

Generally, yoghurt is being perceived as a kind of healthy food which has a low fat and essential value in terms of protein and vitamins. In the past, yoghurt was made at home by simply allowing milk to ferment. Recently, most yoghurt is manufactured by using special lactic acid producing bacteria. Typical production of yoghurt involves a symbiotic association of the starter cultures *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, in a 1:1 ratio (Hewitt et al., 1985; Kiliç, 1999; Akin, 2006). According to the Code of Federal Regulations of the FDA (FDA, 1996), yogurt is defined as the "food produced by culturing one or more of the optional dairy ingredients (cream, milk, partially skimmed milk, and skim milk) with a characterizing bacteria culture that contains the lactic acid-producing bacteria, *L. bulgaricus* and *S. thermophilus*". These bacteria may have probiotic properties and they have a long history of safe use as starter culture bacteria (Katla et al., 2001). Yoghurt production can result from the use of starter cultures derived from a previous yoghurt batch or from inoculation of milk with a commercially prepared culture. Regardless of starter culture origin, the reduced pH of yoghurt should inhibit the growth of acid-sensitive organisms, thus providing the yoghurt with antimicrobial qualities (Bachrouri et al., 2006).

Recently, increased focus has been given to food as potential vehicles of antimicrobial substances. These foods have become an important health care sector in most countries. Among them, the fermented dairy products, especially yoghurt, are a classic example of traditional food originating from the Middle East and Eastern Mediterranean, and they play important role in safe health (Tamime et al., 2006; Kyriacou et al., 2008).

Antimicrobial substances are produced by a wide range of bacteria, including dairy starter cultures. Lactic acid bacteria (LAB) can produce antimicrobial substances with the capacity to inhibit the growth of pathogenic and

*Corresponding author E-mail: oktay.yerlikaya@ege.edu.tr. Tel: +90 232 311 27 25. Fax: +90 232 388 17 64.

spoilage microorganisms.

Lactic acid, acetic acid, formic acid, phenyllactic acid, caproic acid, organic acids, ethanol, hydrogen peroxide, diacetyl, bacteriocins, reuterin, reutericyclin and bactericidal proteins are included among these compounds. (Marshal and Tamime, 1997; Leroy et al., 2004; Savadogo et al., 2004; Herreros et al., 2005; Tamime et al., 2007; Gugliemotti et al., 2007; Fadela et al., 2008; Yerlikaya and Kesenka , 2009; Šuškovi et al., 2010). Particularly lactic acid and acetic acid are important compounds, inhibiting a broad range of microorganisms (Helander et al., 1997)

Especially, yoghurt bacteria are effective for the prevention and treatment of some diseases related with pathogen microorganisms by several mechanisms such as production of substances above mentioned which are active at low pH. In addition, they appear to be the elimination of colonization with pathogenic bacteria and the treatment of gastrointestinal tract infections, including those sustained by *Clostridium difficile* and *Helicobacter pylori* (Petti et al., 2008).

Many LAB are resistant to antibiotics. This resistance attributes are often intrinsic and nontransmissible. On the other hand, intrinsically antibiotic-resistant strains may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to the administration of various antimicrobial agents (Erdogrul and Erbilir, 2006).

Although, there is a lot of research about antimicrobial activity and antibiotic resistance of LAB, few of them have been studied in isolation from homemade yoghurts.

Therefore, the aim of this study was to investigate the effect of antimicrobial activity and antibiotic resistance of yoghurt bacteria which were isolated and identified from yoghurt samples collected from several regions of Turkey. These yoghurt bacteria were tested for their antimicrobial activities against Gram-positive and Gram-negative food pathogenic and spoilage microorganisms. Their resistances towards some antibiotics were also observed.

MATERIAL AND METHODS

Samples

A total of 30 different homemade yoghurts collected from several regions of Turkey. All samples were transferred to the laboratory under refrigeration and stored at 4°C until their analysis.

Isolation of lactic acid bacteria

The yoghurt samples (10 g) were diluted in 90 ml Ringer's solution (Merck, Darmstadt, Germany), homogenized by stomacher and serially diluted with Ringer's solution. Man Ragosa Sharpe Agar (MRS, Merck, Darmstadt, Germany) were used for isolation of *L. bulgaricus*, M17 Agar (Merck, Darmstadt, Germany) and ST Agar (*S. thermophilus* agar) were used for isolation of *S. thermophilus*. Also, LS-Differential Medium (Fluka, BioChemika) with added triphenyl tetrazolium chloride (Merck, Darmstadt, Germany) was used for isolation of both *L. bulgaricus* and *S. thermophilus*. This additional medium was used to obtain better colony formation from these bacteria. LS-Differential Medium and MRS agar plates were incubated anaerobically at 42°C for 72 h by using Anaerocult A (Merck, Darmstadt, Germany) for *L. bulgaricus,* M17 and ST-Agar plates were incubated aerobically at 37°C for 72 h for *S. thermophilus*. Appropriate colonies of each bacterial species were randomly taken from the plates of each yoghurt sample. Finally total of 208 strains were isolated.

Characterization and identification

The following characteristics of the bacterial isolates were checked using standard protocols: Simple staining, Gram-staining, cell morphology, catalase reaction, production of gas from glucose, growth at different salt levels (2, 4 and 6.5% NaCl), pH tolerance, reduction of litmus milk and growth at 10, 15, 30, and 45°C. Isolates were screened for their ability to ferment 17 different carbohydrates (glucose, fructose, mannose, lactose, sorbitol, ribose, salicin, threalose, mellibiose, sucrose, mannitol, arabinose, maltose, raffinose, galactose, xylose and rhamnose).

For determination of some enzymatic reactions and carbohydrate fermentation tests of *Lactobacilli* and *Streptococci* isolates, API 50CH strips with CHL medium, API 20 Strep with GP medium was used respectively according to manufacturer's instructions (BioMerieux, France). The interpretation of the results was facilitated using Apilab Plus (version 3.3.3; Biomerieux) . Relatively, *Streptococcus* isolates were confirmed with the Vitek 2 compact system (BioMerieux, France).

Antimicrobial activity

Preparation of pathogen and spoilage microorganisms

Foodborne pathogens and spoilage microorganisms (*Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus coagulans*, *Klebsiella pneumoniae* and *Pseudomonas fluorescens)* for testing antibacterial activity were received from the collection Department of Dairy Technology (Ege University, Turkey). Test microorganisms were inoculated to nutrient broth (Merck, Darmstad) and incubated at appropriate incubation conditions until the concentration reached 10^7 to 10^8 cfu/ml.

Preparation of *L. bulgaricus* and *S. thermophilus* strains

L. bulgaricus and *S. thermophilus* strains were activated overnight in MRS and M17 broth, respectively. Activated cultures were added into heat-treated (at 110°C for 10 min) reconstituted skim milk at a ratio of 3%. Samples were incubated for 24 h at 42 and 37°C, respectively.

Determination of antimicrobial activity

Antimicrobial activity of *L. bulgaricus* and *S. thermophilus* were determined by the agar diffusion method according to Tagg and McGiven (1971) and Shahani et al. (1976) with minor modifications. All tests were performed in nutrient agar and nutrient broth (Merck, Darmstadt, Germany).

Suspensions of the test bacteria were added containing 25 ml sterile nutrient agar at 43 to 45ëC, and poured into petri plates (8 cm diameter). Plates were left at 37ëC for 1 h and the paper discs diffusion which are treated with *L. bulgaricus* and *S. thermophilus*

Table 1. Inhibition of some food-borne pathogens and spoliage bacteria by *L. delbrueckii* ssp. *bulgaricus* strains.

Strains	*B. cereus*	*P. flourecens*	*Kl. pnomanie*	*L. monocytogenes*	*B. coagulans*	*S. aureus*	*E. coli*
G61	-	-	+	-	-	-	+
OL4	+	+	-	+	-	-	+
K1L2	+	+	-	+	-	-	+
D6R	-	-	-	-	-	+	+
F5R	+	+	+	+	+	+	+
Z4R	-	+	-	-	-	+	+
FL2	+	-	-	+	-	-	+
OL2	-	-	-	+	-	-	+
OL1	+	+	+	+	-	-	+
SL3	+	-	-	+	-	-	+
G61	+	+	-	+	-	-	+
E3M2	+	+	+	-	-	-	+
E1M4	+	+	+	+	-	-	+
OL2	+	-	+	+	-	-	+
ML1	-	-	+	+	-	-	+
G3R	+	+	+	+	-	-	+
K1L4	+	+	+	+	-	-	+
SL1	+	+	+	+	-	+	+
G44	-	-	+	+	-	+	+
G74	-	+	+	+	-	-	+
OL42	+	+	+	+	-	-	+
P4R	+	+	+	+	-	+	+
G53	-	-	+	-	+	-	+
OL1	+	+	+	-	-	-	+
K2L3	-	+	+	-	+	-	+
Total percent (%)	64	64	68	72	12	24	100

+ : positive, - : negative.

strains was used to detect the antibacterial activity of yoghurt bacteria. Then, plates were left at appropriate temperature for incubation.

After the incubation, inhibition zones formed in the medium were measured in millimeter (mm). Antimicrobial activity was evaluated negative (-) for zone diameters less than 8 mm and positive (+) for zone diameters higher than 8 mm. The experiment was performed in duplicate.

Determination antibiotic susceptibility testing in isolated strains

Antibiotic susceptibility of isolated strains were determined by using VITEC 2 compact GN card system according to the manufacturer's instructions in Veterinary Control and Research Institute, Turkey. The antibiograms have included bacitracin (0.0006 mg), novobiocin (0.000075 mg), polymixin-B (0.00093 mg), and optochin (0.000399 mg) in this system.

RESULTS AND DISCUSSION

Antimicrobial activity

A total of 25 *L. bulgaricus* and 16 *S. thermophilus* strains were tested for their antimicrobial activity against foodborne pathogens and spoilage bacteria such as *L. monocytogenes*, *B. cereus*, *S. aureus*, *E. coli*, *B. coagulans*, *K. pneumoniae* and *P. fluorescens.*

The antimicrobial activity of *L. bulgaricus* strains are given in Table 1. All of *L. bulgaricus* strains exhibited antimicrobial activity against to *E. coli* (Figure 1). Also, among these 25 strains, only strain F5R has shown antimicrobial activity against all of the test bacteria. The antimicrobial activity of *S. thermophilus* strains are given in Table 2. All of *S. thermophilus* strains presented antimicrobial activity against to *K. pneumoniae* (Figure 2). Two strains of *S. thermophilus* (SL4 and SY2) had antimicrobial activity against all test bacteria. Moreover the second highest antimicrobial activity of *L. bulgaricus* and *S. thermophilus* were both against to *L. monocytogenes* (Figure 3).

It has been known that *L. bulgaricus* has a preservative effect on the product not only because of the production of lactic acid and hydrogen peroxide, but also by the help of the antimicrobial compounds (example, bacteriocin) it produces. The compound, namely Bulgarican, is inhibitory towards both Gram-positive and Gram-negative bacteria. Some inhibitory compounds against *Staphylococcus* and *Clostridium* species have also been found (Erkus, 2007).

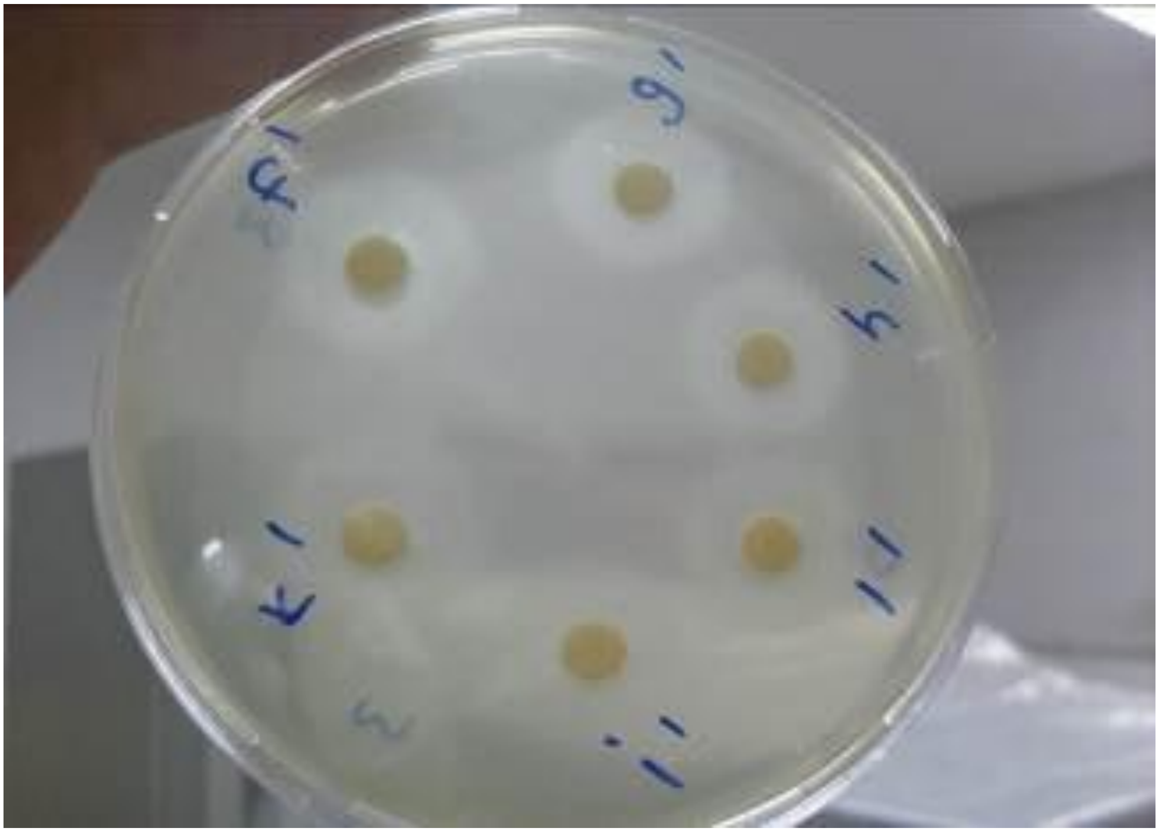

Figure 1. Antimicrobial activity of *L. bulgaricus* and *S. thermophilus* strains against to *E. coli.*

Table 2. Inhibition of some food-borne pathogens and spoliage bacteria by *S. thermophilus* strains.

Strains	*B. cereus*	*P. flourecens*	*Kl. pnomanie*	*L. monocytogenes*	*B. coagulans*	*S. aureus*	*E.coli*
E2MN2	−	+	+	+	+	−	+
K1M6	−	+	+	+	−	−	+
K1M4	−	+	+	+	+	−	+
K2M4	−	+	+	+	+	−	+
K2M42	−	−	+	+	+	−	+
SY72	−	+	+	+	−	+	+
C6	−	+	+	+	−	+	+
SL4	+	+	+	+	+	+	+
SY(2)	+	+	+	+	+	+	+
M3	−	+	+	+	+	+	+
ET6	−	−	+	+	+	+	+
K2M1	−	−	+	−	−	−	−
K2M7	−	−	+	+	−	−	−
SY73	−	+	+	−	−	+	−
C1M	−	−	+	−	+	−	+
F1M	−	−	+	+	−	+	−
K2M1	−	+	+	−	−	−	+
MN3	−	−	+	+	−	−	−
Total percent (%)	11	61	100	77	50	39	72

+ : positive, - : negative.

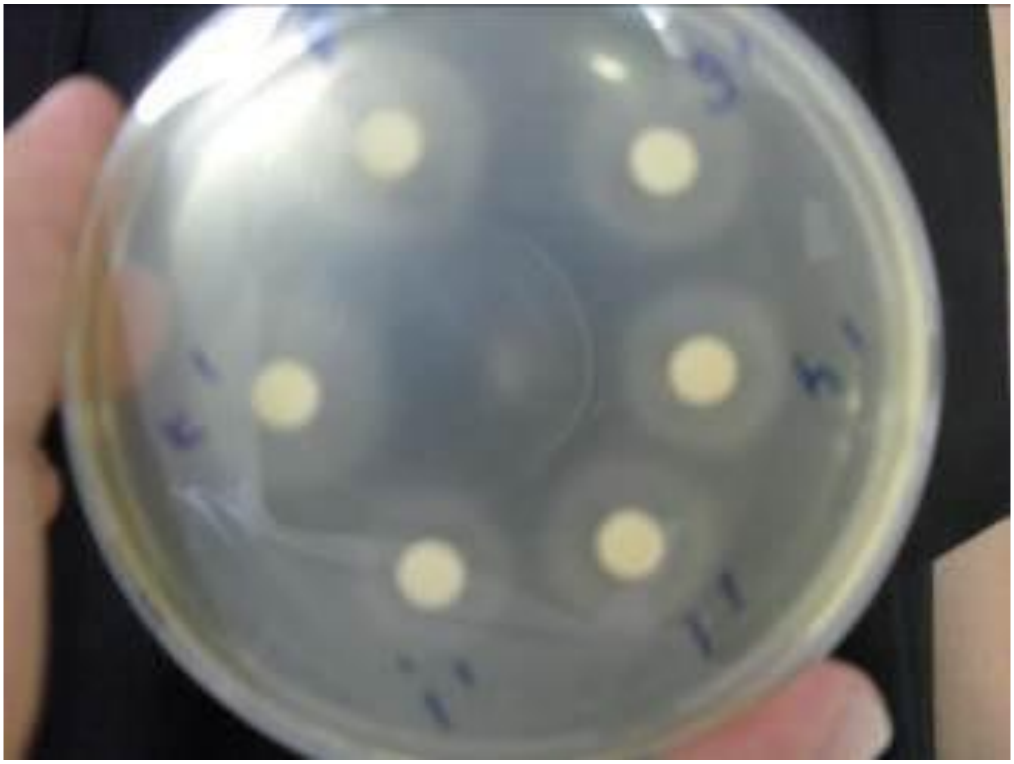

Figure 2. Antimicrobial activity of *L. bulgaricus* and *S. thermophilus* strains against to *K. pneumoniae.*

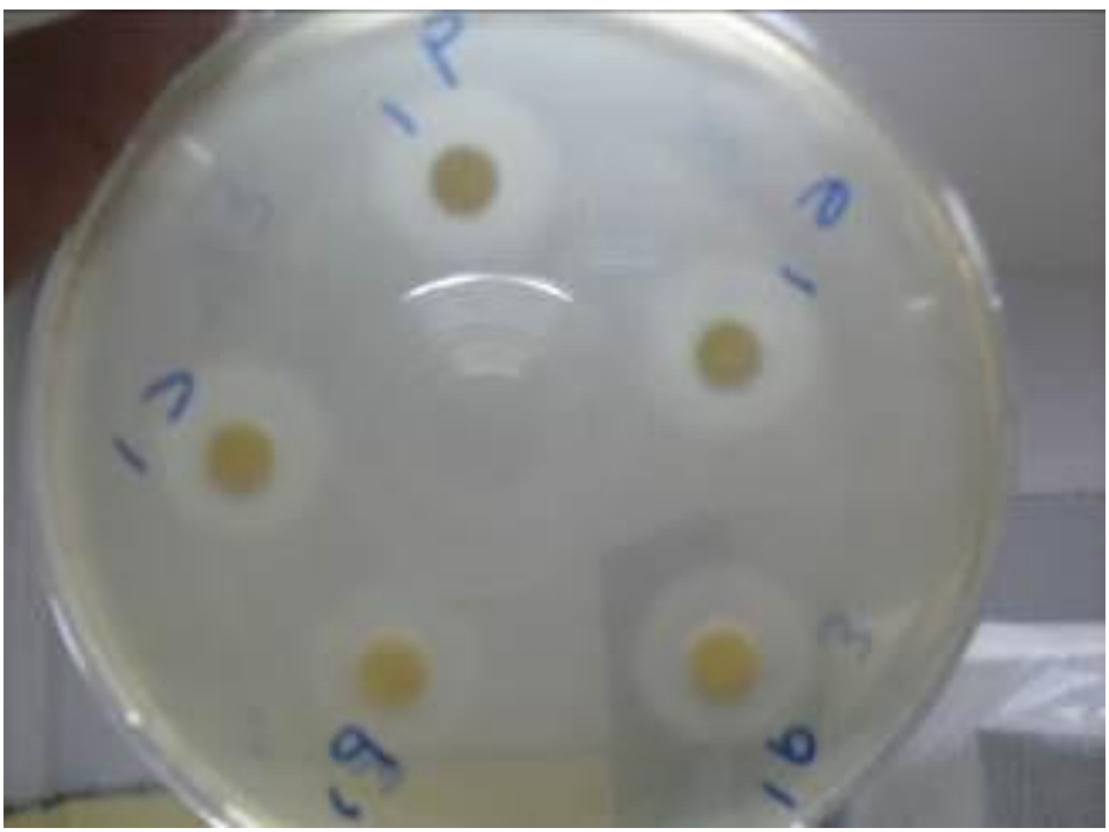

Figure 3. Antimicrobial activity of *L. bulgaricus* and *S. thermophilus* strains against to *L. monocytogenes.*

Mezaini et al. (2009) reported that twenty LAB strains which were isolated from Algerian dairy milk were screened for their antagonistic activity against *Listeria innocua*, *Enterococcus faecalis*, *B. cereus*, *Bacillus subtilis*, *S. aureus*, *Staphylococcus epidermitidis*, *E. coli,* and *Salmonella typhimurium*. Among these bacteria *S. thermophilus* T2 strain showed a wide inhibitory spectrum against all the Gram positive target bacteria used in this study except against *S. aureus*. In addition, *S. thermophilus* T2 did not show any inhibitory activity against *E. coli* and *S. typhimurium*.

Some researcher found that the antimicrobial effect of *Lactobacillus* ssp. have been higher than *Streptococcus* ssp. (Yuksekdag and Beyatli, 2003). Especially, *L. bulgaricus* has shown antimicrobial activity against *S. aureus,* because it consists of hydrogen peroxide production (Dahiya and Speck, 1967). Also, *L. bulgaricus* produces antimicrobial substance different from lactic acid and these substances are effective against to *S. aureus* (Abdel-Bar et al., 1987).

Mel'nikova and Koroleva (1975) have proved the antibacterial effect of *S. thermophilus* to *E. coli*, *Salmonella,* and *Pseudomonas* species. However, effect of *L. bulgaricus* have further revealed. Researchers have noted that this effect was derived from *L. bulgaricus* features producing more advanced lactic acid.

Kiliç (1990) found a weak antibacterial effect of 12 *L. bulgaricus* strains against *B. cereus* strains. Arslan (2002) has identified the species strains of *L. bulgaricus* and *S. thermophilus* with morphological and biochemical tests, and their antimicrobial activity on *L. monocytogenes*, *C. difficile*, *E. coli 0157: H7* and *Staph. aureus*, were determined by agar-disc diffusion method. This study also supported the aforementioned information, which stipulated that all yogurt culture showed antimicrobial effect against *E. coli*.

Lactic acid bacteria may have probiotic properties and they have a long history of safe use as starter culture bacteria (Salminen et al., 1998). Therefore, these bacteria have not received much attention in regard to antimicrobial resistance. However, since starter culture bacteria are added to different kinds of food and intentionally grown into high numbers, such bacteria could represent a potential source for spread of antimicrobial resistance genes (Katla et al., 2001).

Antibiotic resistance

Antibiotic resistance of *L. bulgaricus* strains which were isolated from homemade yoghurt samples are presented in Table 3. As it was seen, none of *L. bulgaricus* strains exhibited resistance to the polymyxin-B. Some of *L. bulgaricus* strains were resistant towards only optochin but some strains (F5R, E1M4, SL1, D6R, E3M2, and FL2) were resistant to both optochin and novobiocin. Only the strain OL4 was found to be resistance towards novobiocin, optochin and bacitracin.

Kyriacou et al. (2008) detected that *Lactobacillus* ssp. were not resistant against tetracycline, chloramphenicol or rifampicin, but these strains were susceptible to erythromycin. Charteris et al. (1998) reported that all the strains of *L. bulgaricus* were susceptible to bacitracin. Erdo rul and Erbilir (2006) reported that *L. bulgaricus* isolate was susceptible to all of the antibiotics. Sozzi and Smiley (1980) reported that most *L. bulgaricus* strains were resistant to polymyxin-B. *L. bulgaricus* strains also show an increase in the numbers of strains resistant to polymyxin-B and novobiocin.

When a comparison was done with the results reported by the aforementioned research, this study would therefore appear to show that *L. bulgaricus* strains are resistant to the mentioned antibiotics.

S. thermophilus is usually sensitive to chloramphenicol, tetracycline, erythromycin, cephalothin quinupristin/dalfopristin and ciprofloxacin. In contrast, it shows moderate to high resistance to aminoglycosides (gentamicin, kanamycin, and streptomycin), trimethoprim and sulphadiazine (Ammor et al., 2007). Among the different species included in the genus *Streptococcus*, only *S. thermophilus* is of technological importance. However, few data are available on the antibiotic susceptibility of this species.

Antibiotic resistance of *S. thermophilus* strains which were isolated from homemade yoghurt samples are summarized in Table 4. It was noticed that most of the *S. thermophilus* strains were not found to be resistant towards tested antibiotics.

While some strains of *S. thermophilus* like C6 and SL4 exhibited resistance to novobiocin, SY72, M3, C1M, and F1M were shown to optochin. Strains ET6 and SY73 were found to be resistant in both novobiocin and optochin.

Sozzi and Smiley (1980) examineted the antibiotic profile of yoghurt starter cultures, and 15 strains of *S. thermophilus* were tested against several antibiotics.

Examined strains were found to have varying resistance levels to polymyxin-B. While Kyriacou et al. (2008) did not find any resistant *S. thermophilus* strains to antibiotics, Lim et al. (1995) found that *S. thermophilus* was susceptible to antibiotics.

Conclusion

This study shows that, some *L. bulgaricus* and *S. thermophilus* strains which were isolated from homemade yoghurts had shown antimicrobial activity against some foodborne pathogen and spoilage microorganisms especially *L. monocytogenes*, *E. coli*, *K. pneumonie* and *P. fluorescens*. Also, in some of *L. bulgaricus* and *S. thermophilus* strains had determinated resistance to some antibiotics such as novobiocin and optochin.

Our results show that certain strains which would be

Table 3. Antibiotic resistance of some food-borne pathogens and spoliage bacteria of *L. delbrueckii* ssp. *bulgaricus* strains.

Strains	Novobiocin (0.000075 mg)	Optochin (0.000399 mg)	Bacitracin (0.0006 mg)	Polymixin-B (0.00093 mg)
G61	-	-	-	-
OL4	+	+	+	-
K1L2	-	-	-	-
D6R	+	+	-	-
F5R	+	+	-	-
Z4R	-	+	-	-
FL2	+	+	-	-
OL2	-	+	-	-
OL1	-	-	-	-
SL3	-	-	-	-
G61	-	-	-	-
E3M2	+	+	-	-
E1M4	+	+	-	-
OL2	+	+	-	-
ML1	-	-	-	-
G3R	-	-	-	-
K1L4	-	-	-	-
SL1	+	+	-	-
G44	-	-	-	-
G74	-	-	-	-
OL42	+	+	-	-
P4R	+	+	-	-
G53	-	-	-	-
OL1	+	+	-	-
K2L3	-	+	-	-
Total percent (%)	44	56	4	0

+ : positive, - : negative.

Table 4. Antibiotic resistance of some food-borne pathogens and spoliage bacteria of *S. thermophilus* strains.

Strains	Novobiocin (0.000075 mg)	Optochin (0.000399 mg)	Bacitracin (0.0006 mg)	Polymixin-B (0.00093 mg)
E2MN2	-	-	-	-
K1M6	-	-	-	-
K1M4	-	-	-	-
K2M4	-	-	-	-
K2M42	-	-	-	-
SY72ST	-	+	-	-
C6	+	-	-	-
SL4LSD	+	-	-	-
SY(2)	-	-	-	-
M3	-	+	-	-
ET6	+	+	-	-
K2M1	-	-	-	-
K2M7	-	-	-	-
SY73	+	+	-	-
C1M	-	+	-	-
F1M	-	+	-	-
K2M1	-	-	-	-
MN3	-	-	-	-
Total percent (%)	12	33	0	0

+ : positive, - : negative.

used in the manufacture of homemade yoghurts using natural starters have various antimicrobial activity and antibiotic resistances. Therefore, in yogurt manufacturing, we consider it prudent to use strains. Also, there is need for more research on antibiotic resistance profiles of yoghurt bacteria. Further research will be focus on varied antibiotic resistance of *L. bulgaricus* and *S. thermophilus* strains isolated from homemade yoghurts.

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