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Safety Assessment of Dairy Microorganisms, *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, Isolated from Traditional Yoghurt Cultures

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ABSTRACT

The traditional fermented food consumption has become very popular because of the increasing public concern about food additives. Lactic Acid Bacteria (LAB) species have traditionally been used as starter cultures in the production of fermented food. LAB can acquire antibiotic resistance from other bacteria in the natural environment and different resistant mechanisms via mutation. The resistance of bacteria to antibiotics is an increasingly important public health problem worldwide. In this study, antibiotic resistance of 115 *Streptocoocus thermophilus* and 35 *Lactobacillus bulgaricus* isolates obtained from traditional Turkish yogurts were tested against kanamycin, chloramphenicol, erythromycin, ampicillin, rifampicin, tetracycline, vancomycin and gentamicin using disc diffusion method. Study results showed that most strains were susceptible to all the antibiotics tested while a few of them were determined to be resistant only to kanamycin, ampicillin, erythromycin, and tetracycline. When contacted in a human body, resistant strains might transfer the related genes to the pathogenic species, which may result in devastating consequences.

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Introduction

Lactic Acid Bacteria (LAB) species have traditionally been used as starter cultures in the production of fermented food such as cheese, butter and yogurt. Considering the long history of their presence and use in traditional fermented food, LAB have been presented in the status of ‘‘Generally Regarded as Safe’’ (GRAS) by the American Food and Drug Agency. LAB species are also commonly found among the resident microbiota of the gastrointestinal tract (GIT) of vertebrates (Carr et al., 2002). However, an increasing concern has arisen about multi antibiotic resistance features of bacteria in the light of the current knowledge.

Evaluation of antibiotic resistance in bacteria is mainly based on two factors; the presence of resistance genes and the selective pressure by the use of antibiotics (Levy and Marshall, 2004). The presence of intrinsic and acquired antibiotic resistance genes in LAB do not bear a significant clinical risk. Conversely, the possibility that food safety cultures might transfer antibiotic resistance genes to pathogenic opportunistic species either during food manufacture or during passage through the gastrointestinal tract (GIT) is disquieting (Salyers et al., 2004; Ammor et al., 2007). Resistances by mutation are assumed as a low risk of horizontal spread, while acquired resistances mediated by the addition of genes introduce the real danger of transfer among the species (Normark and Normark, 2002). It is possible for LAB to acquire antibiotic resistances from other bacteria in the natural environment as well as distinct resistant mechanisms through mutations. Plasmid transfer must be considered as a system which has the potential to generate antibiotic resistant pathogenic bacteria. LAB used in starter cultures can capture antibiotic resistance genes from other bacteria with the aid of conjugative plasmids and transposons. It has been demonstrated that when antibiotic resistant gene carrier LAB strains are used as starters, these resistant genes could be mobilized and transferred to other bacteria including pathogens (Fraqueza, 2015).

Antibiotics kill or inhibit susceptible bacteria; however resistance bacterial genus or species carrying inherent (natural) or acquired genes remain unaffected (Normark and Normark, 2002). Generating resistance against antibiotics in originally susceptible microorganisms has become a major threat to public health (Mazel and Davies, 1999). The resistance of bacteria to antibiotics is an increasingly important public

health problem worldwide. It has been reported that in 11 European countries, antibiotic resistance is correlated with antibiotic use (Bronzwaer et al., 2002). Decades of antibiotic use have resulted in bacterial resistance to many known antibiotics. Each year, in world, antibiotic-resistant bacteria sicken many people, and most of them die as a direct result of these infections. The food chain has been recognized as one of the main ways of transmission of antibiotic resistance from pathogens to potential starter bacterial population (Teuber et al., 1999).

The traditional fermented food consumptions have become very popular issue reflecting food additives. On the other hand, consumers do not consider antibiotic resistance features and safety aspects of traditional starter. Besides, non-commercial strains might be a rich reservoir of unique genetic material (Petrova and Gouliamova, 2006). LAB isolated from chicken resistance to the familiar antibiotics used in the farm (Shazali et al., 2014) and *S. thermophilus* isolates carrier for antibiotic resistance determinants in commercial cheese (Wang et al., 2006). S*treptococcus thermophilus* and *Lactobacillus bulgaricus* are non-pathogenic organisms and are important LAB strains in yoghurt industry. The 115 *S. thermophilus* and 35 *Lb. bulgaricus* strains evaluated here were originally isolated from traditional yoghurts in our previous study (Gezginc et al., 2015). The aim of this study was the molecular identification of a large collection of LAB strains from traditional yoghurts and to assess their antibiotic resistance features thus to reveal the potential risks of these isolates in terms of food safety when used as starters.

Material and Method

Molecular Identification of Strains

A total of 115 *Streptocoocus thermophilus* and 35 *Lactobacillus bulgaricus* species used in this study were isolated from yoghurt in a previous study (Gezginc et al., 2015). *S. thermophilus* isolates were grown in 1 % (w/v) sucrose containing M17 medium at 42°C and *Lb. bulgaricus* isolates were grown in MRS medium at 37°C. DNA templates extracted from a single bacterial colony do not require further purification after being boiled and can be directly used for PCR amplification. Molecular identification of the strains was performed with 16S rRNA region sequencing. Bacterial domain specific 365F (ACWCCTACGGGWGGCWGC) and 1064R (AYCTCACGRCACGAGCTGAC) primers (Winsley et al., 2012) were purchased from Iontek (Istanbul, Turkey). Polymerase chain reaction was performed in 40 µL by using 5 U/µL DNA polymerase (Thermo Scientific, Waltham, MA) and 10 × PCR buffer (Thermo Scientific). A total of 20 pmol of each primer was used, and deoxynucleoside triphosphates (Thermo Scientific) were used at a concentration of 250 µM for each. The PCR amplification program consisted of 1 cycle of 95°C for 5 min, 30 cycles of 95°C for 30 s, 54°C for 1 min, 72°C for 1.5 min and 1 cycle of 72°C for 7 min. The amplification products (746 bp) were then separated by electrophoresis in 1.5% (w/v) agarose gel. Ethidium bromide (0.5 µg/ml) staining was utilized to visualize the amplicons under UV transillumination. The amplified PCR products were cleaned using the Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA) and were sent to Iontek (Istanbul, Turkey) for nucleotide sequencing. Observed sequence data were compared GenBank database using the basic local alignment search tool (BLAST) provided by the National Center for Biotechnology Information (NCBI) public domain.

Antibiotics Susceptibility Testing

Antibiotic features of the identified *S. thermophilus* and *Lb. bulgaricus* isolates were tested by disc diffusion method on SM17 and MRS agar plates, correspondingly. All isolates were screened for their susceptibility to kanamycin, chloramphenicol, erythromycin, ampicillin, rifampicin, tetracycline, vancomycin and gentamicin. Appropriate antibiotic stock concentration was made in 5 mL medium and then working dilution series were prepared from stock solution (Table 1). To prepare antibiotic discs, whatman filter paper No. 3 was cut using puncher (5mm in diameter) and disc paper was sterilized in an autoclave. 20 µL of working antibiotics solution (each antibiotic at its pre-determined concentration) were added on sterile filter paper discs using a pipette and the discs were dried in laminar flow cabinet. Once dried, they were stored in sealed tubes at minus 20°C until used. In order to determine the antibiotic resistances, the plates were inoculated with 100 µL of an individual isolate at 10^5 colony-forming unit (cfu)/mL concentration. Then five discs containing the antibiotic at different concentrations were placed onto the overlaid plates and incubate at 37°C (*Lb. bulgaricus*) and 42°C (*S. thermophilus*). The diameters of inhibition halos (Minimum Inhibitory Concentration (MIC)) were measured in mm.zone (Figure 1) and experiment were performed in triplicate.

Table 1 Antibiotics used in this study.

Antibiotics	Stock Concentration (mg/mL)	Tested Min-Max Concentration (µg/mL)
Kanamycin	5 mg/mL; dissolved in dH_2O	0.625 - 5
Chloramphenicol	5 mg/mL; dissolved in absolute ethanol	0.625 - 5
Erythromycin	5 mg/mL; dissolved in absolute ethanol	1.25- 10
Ampicillin	5 mg/mL; dissolved in dH_2O	1.25- 10
Rifampicin	40 mg/mL; dissolved in 98% methanol	12.5- 100
Tetracycline	5 mg/mL; dissolved in absolute ethanol	0.625 - 5
Vancomycin	8 mg/ml; dissolved in dH_2O	1- 8
Gentamicin	50 mg/ml; dissolved in dH_2O	6.25-50

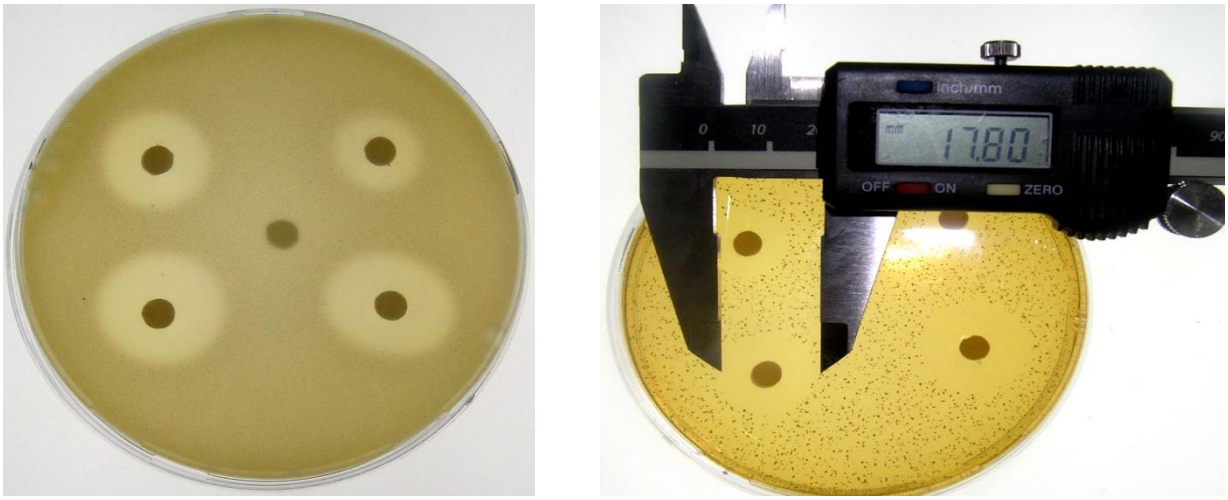

Figure 1 Determination and measurement of antibiotic inhibition zone

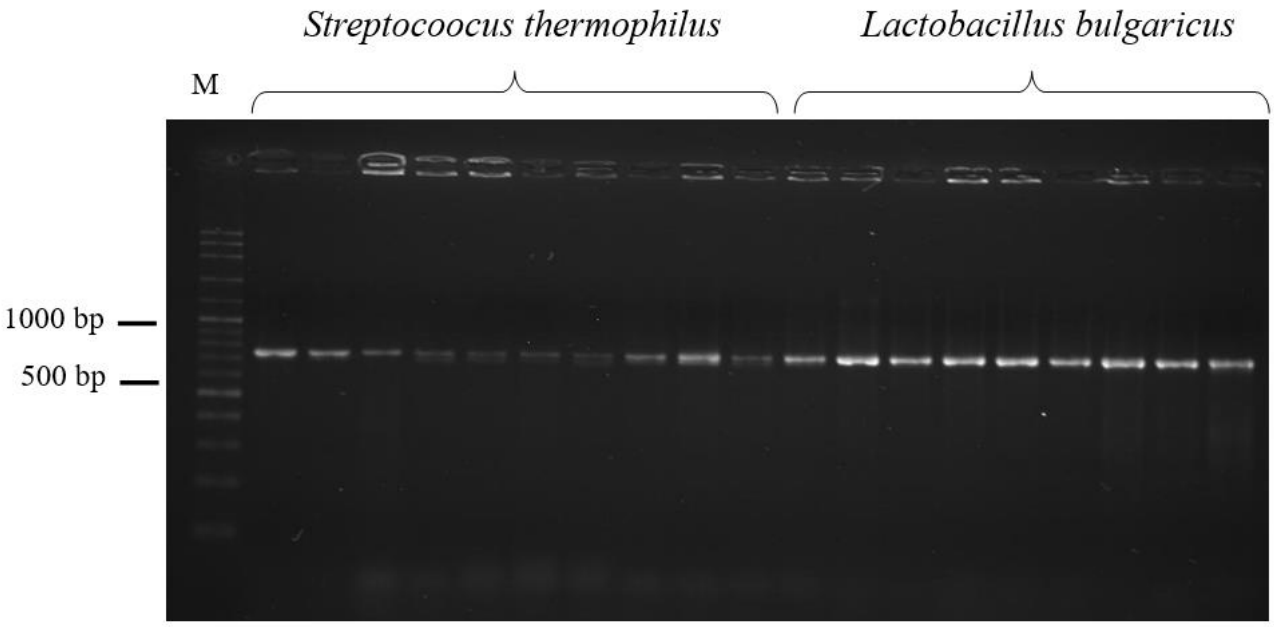

Figure 2 PCR amplification of 16S rDNA region for *Streptococcus thermophilus* and *Lactobacillus bulgaricus* isolates. M: DNA ladder

Results and Discussion

Molecular Identification of Isolates

The PCR amplification of 16S rDNA by using universal 365F and 1064R primers produced a single band at the expected size which was obtained with a 54°C annealing temperature. The length of the PCR fragment was approximately 746 bp (Figure 1). The amplified 16S rDNA fragments were sequenced in order to verify the accuracy of isolate identification.16S rDNA fragment sequence of each isolate was read in three replications using either forward or reverse primer. Nucleotide alignments were constructed using Clustal X (Thompson et al., 1997) and a single consensus sequence was generated. The consensus sequences of PCR fragments generated high percent matches (99 -100%) with those of the corresponding genes from the genome database.

Antibiotic Resistance of Isolates

Minimal inhibitory concentration (MIC) values for all bacterial isolates were determined using liquid medium and observed MIC of used antibiotics were given in Table 1. Identified 150 isolates (*S. thermophilus* (115) and *Lb. bulgaricus* (35)) have been submitted to the antibiotic susceptibility test for eight different antibiotics. Each antibiotic was tested at four different concentrations level and dissolved liquid (dH_2O or ethanol) was used as control. Antibiotics resistance or susceptible profile of *S. thermophilus* and *Lb. bulgaricus* isolates were determined according to diameter of inhibitory zone. Depending on the type of antibiotic and its concentration, numbers of the resistance strains of *S. thermophilus* and *Lb. bulgaricus* were determined and the results were presented in Table 2 and 3. Yoghurt bacteria are generally susceptible to antibiotics inhibiting the synthesis of protein such as chloramphenicol, erythromycin, and tetracycline, and more resistant to aminoglycosides (kanamycin, and gentamicin).

Table 2 Distribution of MICs of kanamycin, chloramphenicol, erythromycin, ampicillin, rifampicin, tetracycline, vancomycin and gentamicin for *Streptococcus thermophilus* isolates.

Antibiotics	Concentrations µg/ml	Diameter of inhibition zone (mm)						
		5*	6-11	12-17	18-23	24-29	30-35	≥36
Kanamycin	0.625	49 (43)	44 (38)	17 (15)	2 (2)	1 (1)	1 (1)	1 (1)
	1.25	26 (23)	51 (44)	27 (23)	8 (7)	1 (1)	1 (1)	1 (1)
	2.5	21 (18)	23 (20)	54 (47)	14 (12)	1 (1)	1 (1)	1 (1)
	5	19 (17)	12 (10)	48 (42)	31 (27)	3 (3)	1 (1)	1 (1)
Chloramphenicol	6.25×10^{-4}	26 (23)	59 (51)	23 (20)	4 (3)	1 (1)	1 (1)	1 (1)
	12.5×10^{-4}	9 (8)	37 (32)	53 (46)	10 (9)	4 (3)	1 (1)	1 (1)
	25×10^{-4}	4 (3)	11 (10)	67 (58)	24 (21)	7 (6)	1 (1)	1 (1)
	50×10^{-4}	2 (2)	4 (3)	39 (34)	51 (44)	14 (12)	4 (3)	1 (1)
Erytromycin	12.5×10^{-4}	23 (20)	12 (10)	37 (32)	33 (29)	7 (6)	2 (2)	1 (1)
	25×10^{-4}	20 (17)	7 (6)	21 (18)	42 (37)	20 (17)	3 (3)	2 (2)
	50×10^{-4}	17 (15)	6 (5)	11 (10)	37 (32)	33 (29)	9 (8)	2 (2)
	100×10^{-4}	13 (11)	5 (4)	10 (9)	26 (23)	39 (34)	15 (13)	7 (6)
Ampicillin	1.25×10^{-4}	24 (21)	10 (9)	37 (32)	27 (23)	13 (11)	3 (3)	1 (1)
	2.5×10^{-4}	19 (17)	3 (3)	29 (25)	37 (32)	14 (12)	9 (2)	4 (3)
	5×10^{-4}	16 (14)	3 (3)	17 (15)	34 (30)	27 (23)	10 (9)	8 (7)
	10×10^{-4}	11 (10)	8 (7)	11 (10)	26 (23)	31 (27)	13 (11)	15 (13)
Rifampycin	12.5×10^{-4}	17 (15)	7 (6)	43 (37)	19 (17)	14 (12)	14 (12)	1 (1)
	25×10^{-4}	10 (9)	5 (4)	28 (24)	31 (27)	16 (14)	15 (13)	10 (9)
	50×10^{-4}	9 (8)	1 (1)	19 (17)	36 (31)	23 (20)	16 (14)	11 (10)
	100×10^{-4}	8 (7)	1 (1)	11 (10)	34 (30)	24 (21)	14 (12)	23 (20)
Tetracycline	6.25×10^{-4}	19 (17)	14 (12)	43 (37)	30 (26)	7 (16)	1(1)	1(1)
	12.5×10^{-4}	16 (14)	6 (5)	32 (28)	35 (30)	22 (19)	3 (3)	1(1)
	25×10^{-4}	12 (10)	4 (3)	21 (18)	31 (27)	29 (25)	17 (15)	1(1)
	50×10^{-4}	10 (9)	2 (2)	13 (11)	25 (22)	39 (34)	19 (17)	7(6)
Vankomycin	6.25×10^{-2}	19 (17)	21 (18)	63 (55)	8 (7)	2 (2)	1 (1)	1 (1)
	12.5×10^{-2}	17 (15)	13 (11)	51 (44)	26 (23)	6 (5)	1 (1)	1 (1)
	25×10^{-2}	9 (8)	3 (3)	49 (43)	43 (37)	4 (3)	6 (5)	1 (1)
	50×10^{-2}	18 (16)	1 (1)	22 (19)	53 (46)	14 (12)	6 (5)	1 (1)
Gentamicin	12.5×10^{-2}	20 (17)	36 (31)	44 (38)	11 (10)	2 (2)	1 (1)	1 (1)
	25×10^{-2}	17 (15)	5 (4)	49 (43)	37 (32)	5 (4)	1 (1)	1 (1)
	50×10^{-2}	13 (11)	6 (5)	33 (29)	39 (34)	21 (18)	2 (2)	1 (1)
	1	8 (7)	8 (7)	12 (10)	43 (37)	37 (32)	5 (4)	2 (2)

*Disk diameter

Kanamycin resistance of *S. thermophilus* and *Lb. bulgaricus* isolates tested using various concentrations (0.625, 1.25, 2.5 and 5 mg/mL) of the antibiotics on fixed discs. When 0.625, 1.25, 2.5 and 5 mg/mL kanamycin concentrations were used, the percentage of resistant isolates was determined as 43, 23, 18 and 17% respectively in *S. thermophilus* isolates. Same concentrations (0.625, 1.25, 2.5 and 5 mg/mL) were applied to *Lb. bulgaricus* isolates and the resistance ratios were determined as 80, 74, 66 and 54% respectively. A lower percentage (17%) of *S. thermophiles* isolates were resistant to 5 mg/mL of kanamycin compared with *Lb. bulgaricus* isolates which exhibited 54% resistance for the same concentration of it. Antibiotic treatments with decreased kanamycin concentrations showed that the susceptible isolates were low in frequency. Additionally, the disc diffusion may not be accurate for detecting low concentration of antibiotics.

When various concentration of chloramphenicol applied to *S. thermophilus* and *Lb. bulgaricus* isolates, the results showed that 2% and 43% of isolates were resistant for 5 mg/mL chloramphenicol, respectively. Measured inhibition zone indicated that some isolates were susceptible to lower chloramphenicol concentrations as well. These findings agree with published data (Florez et al., 2005).

In another set up, erytromycin was applied in various concentration (100-12.5×10^{-4} mg/mL) to both species. In *S. thermophilus* isolates, resistant ratio to minimum and maximum concentrations used were determined as 11% and 20%, respectively. In *Lb. bulgaricus* isolates, resistant ratio to minimum and maximum concentrations, however, were 43% and 66% in respect to their order. Erytromycin susceptible isolates were found in both species.

Ampicillin concentration was used between 1.25- 10 µg/mL, and *S. thermophilus* and *Lb. bulgaricus* isolates were found to be 10% and 39% resistant for the maximum applied antibiotic dose, respectively. Ampicillin breakpoint was given as 4 µg/mL (Ammor et al., 2007) and some isolates were resistant more than this breakpoint. Rifampicin was dissolved in 40 mg/mL and four dilution concertations (Table 2) were applied. Resistance rateof *S. thermophilus* and *Lb. bulgaricus* isolates were determined as 7% and 31% at 40 µg/mL Rifampicin. When tetracycline antibiotics used at its highest concentration (40 µg/mL), 9% of *S. thermophilus* and 46% of *Lb. bulgaricus* strains were detected as resistant. Stock solution of Vancomycin and Gentamicin

were made in 8 µg/mL and 40 µg/mL concentrations and resistant strains were determined to occupy16% and 7% of *S. thermophilus isolates*, respectively. The percentages of resistant *Lb. bulgaricus* isolates were determined as 31% and 46%. These observations were in accordance with a pervious report through which 34 *S. thermophilus* strains isolated from Turkish yoghurts and their antibiotic resistance patterns were examined. The authors declared that most strains of *S. thermophilus* they studied were found to be resistant to gentamicin (79%) (Aslim et al., 2004).

Table 3 Distribution of MICs of kanamycin, chloramphenicol, erythromycin, ampicillin, rifampicin, tetracycline, vancomycin and gentamicin for *Lactobacillus bulgaricus* isolates.

Antibiotics	Concentrations	Inhibition halo (mm)						
	µg/ml	5*	6-11	12-17	18-23	24-29	30-35	≥36
Kanamycin	0.625	28 (80)	2 (6)	1 (3)	1 (3)	1 (3)	1 (3)	1 (3)
	1.25	26 (74)	4 (11)	1 (3)	1 (3)	1 (3)	1 (3)	1 (3)
	2.5	23 (66)	4 (11)	3 (9)	2 (6)	1 (3)	1 (3)	1 (3)
	5	19 (54)	3 (9)	6 (17)	3 (9)	2 (6)	1 (3)	1 (3)
Chloramphenicol	6.25×10^{-4}	26 (74)	2 (6)	3 (9)	1 (3)	1 (3)	1 (3)	1 (3)
	12.5×10^{-4}	22 (63)	2 (6)	7 (20)	1 (3)	1 (3)	1 (3)	1 (3)
	25×10^{-4}	17 (49)	4 (11)	9 (26)	2 (6)	1 (3)	1 (3)	1 (3)
	50×10^{-4}	15 (43)	1 (3)	3 (3)	12 (34)	2 (6)	1 (3)	1 (3)
Erytromycin	12.5×10^{-4}	23 (66)	3 (9)	3 (9)	2 (6)	2 (6)	1 (3)	1 (3)
	25×10^{-4}	18 (51)	4 (11)	5 (14)	3 (9)	3 (9)	1 (3)	1 (3)
	50×10^{-4}	16 (46)	3 (9)	7 (20)	5 (14)	2 (6)	1 (3)	1 (3)
	100×10^{-4}	15 (43)	6 (17)	4 (11)	5 (14)	3 (9)	1 (3)	1 (3)
Ampicillin	1.25×10^{-4}	23 (66)	5 (14)	3 (9)	1 (3)	1 (3)	1 (3)	1 (3)
	2.5×10^{-4}	20 (57)	6 (17)	4 (11)	2 (6)	1 (3)	1 (3)	1 (3)
	5×10^{-4}	16 (46)	5 (14)	8 (23)	3 (9)	1 (3)	1 (3)	1 (3)
	10×10^{-4}	15 (43)	1 (3)	4 (11)	12 (34)	1 (3)	1 (3)	1 (3)
Rifampycin	12.5×10^{-4}	17 (49)	5 (14)	9 (26)	1 (3)	1 (3)	1 (3)	1 (3)
	25×10^{-4}	14 (40)	2 (6)	13 (37)	3 (9)	1 (3)	1 (3)	1 (3)
	50×10^{-4}	12 (34)	1 (3)	7 (20)	9 (26)	3 (9)	2 (6)	1 (3)
	100×10^{-4}	11 (31)	4 (11)	6 (17)	10 (29)	2 (6)	1 (3)	1 (3)
Tetracycline	6.25×10^{-4}	24 (69)	4 (11)	2 (6)	2 (6)	1 (3)	1 (3)	1 (3)
	12.5×10^{-4}	21 (60)	2 (6)	5 (14)	2 (6)	3 (9)	1 (3)	1 (3)
	25×10^{-4}	17 (49)	4 (11)	8 (23)	2 (6)	2 (6)	1 (3)	1 (3)
	50×10^{-4}	16 (46)	3 (9)	7 (20)	5 (14)	2 (6)	1 (3)	1 (3)
Vankomycin	6.25×10^{-2}	17 (49)	5 (14)	9 (26)	1 (3)	1 (3)	1 (3)	1 (3)
	12.5×10^{-2}	15 (43)	2 (6)	13 (37)	2 (6)	1 (3)	1 (3)	1 (3)
	25×10^{-2}	12 (34)	4 (11)	10 (29)	6 (17)	1 (3)	1 (3)	1 (3)
	50×10^{-2}	11 (31)	8 (23)	3 (9)	7 (20)	4 (11)	1 (3)	1 (3)
Gentamicin	12.5×10^{-2}	25 (71)	4 (11)	1 (3)	2 (6)	1 (3)	1 (3)	1 (3)
	25×10^{-2}	23 (66)	5 (14)	3 (9)	1 (3)	1 (3)	1 (3)	1 (3)
	50×10^{-2}	20 (57)	6 (17)	4 (11)	1 (3)	2 (6)	1 (3)	1 (3)
	1	16 (46)	3 (9)	11 (31)	1 (3)	2 (6)	1 (3)	1 (3)

*Disk diameter *

Conclusion

This study demonstrated that there were large resistance differences between the 150 isolates of two species against the used antibiotics. The origin of antibiotic resistances in the bacteria is unknown, but it is an established fact that LAB can gain antibiotic resistances from other microorganism and mutation. It is a great public concern that commensal bacterial populations from food could act as a reservoir for antibiotic resistance genes. Resistance genes could ultimately be transferred to human pathogens and thereby cause a failure in the treatment of infections. Consequently, foods colonized by the bacteria that harbor such transferable antibiotic resistance genes are becoming a major concern. We suggest that these antibiotic resistance features of the microorganisms must be taken into account when considered as potential starters.

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