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Investigation of the Antibiotic Profiles and Phlogenetic Relationships of the *Lactobacillus* Species Isolated From Goat's and Cow's Milk

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ABSTRACT

Lactobacillus is naturally abundant in raw milk. Lactobacilli may develop antibiotic resistance as a result of unconscious antibiotic usage in animals. The aim of this study was to identify *Lactobacillus* species from raw goat's and cow's milk, investigate phylogenetic relationships, and examine the antibiotic profiles of these strains. In this study, the milk of 10 cows and 5 goats was obtained from some farms in Adana. The Crystal method was used to phenotypically identify different colonies assumed to be *Lactobacillus* that were cultured in milk samples. The disc diffusion test was used to determine their resistance to antibiotics. By using the PFGE method, the phylogenetic relationships of *Lactobacillus* strains were examined. A total of 18 *Lactobacillus* strains were isolated from 10 different cows' milk and 10 *Lactobacillus* strains were identified from 5 distinct goats' milk. When the antibiotic susceptibility profiles of the *Lactobacillus* strains isolated from cow's milk were examined, it was found that all strains were sensitive to vancomycin and chloramphenicol, and 38.9% of them were resistant to some antibiotics. All the *Lactobacillus* strains isolated from goat's milk were shown to be susceptible to ampicillin, vancomycin, chloramphenicol, and 40% of all strains were found to be resistant to some antibiotics. The PFGE analysis showed that 28 *Lactobacillus* strains were separated into 21 pulsetypes, and the strains in the A-B-C-D-E-F-G pulsetypes were found to be 100% similar. Consequently, the sensitivity of *Lactobacillus* species to antibiotics requires more investigation.

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Keçi ve İnek Sütünden İzole Edilen *Lactobacillus* Türlerinin Antibiyotik Profillerinin ve Filogenetik İlişkilerinin Araştırılması

ÖZET

Lactobacillus, çiğ sütte bol miktarda ve doğal olarak bulunur. Hayvanlarda bilinçsiz antibiyotik kullanımı sonucu, laktobasiller antibiyotik direnci geliştirebilir. Bu çalışmanın amacı çiğ keçi ve inek sütünden elde edilen *Lactobacillus* türlerini belirlemek, filogenetik ilişkileri araştırmak ve bu suşların antibiyotik profillerini incelemektir. Çalışmada Adana'daki bazı çiftliklerden 10 inek ve 5 keçi sütü elde edilmiştir. Kristal yöntemi, süt örneklerinde kültürlenen *Lactobacillus* olduğu varsayılan farklı kolonileri fenotipik olarak tanımlamak için kullanıldı. Antibiyotiklere dirençlerini belirlemek için disk difüzyon testi kullanıldı. PFGE yöntemi kullanılarak *Lactobacillus* suşlarının filogenetik ilişkileri incelendi. 10 farklı inek sütünden 18 *Lactobacillus* suşu izole edildi ve 5 farklı keçi sütünden 10 *Lactobacillus* suşu tanımlandı. İnek sütünden izole edilen *Lactobacillus* suşlarının antibiyotik duyarlılık profilleri incelendiğinde, bakterilerin tamamının vankomisin ve kloramfenikole duyarlı olduğu, %38,9'unun bazı antibiyotiklere dirençli olduğu tespit edildi. Keçi sütünden izole edilen tüm *Lactobacillus* suşlarının ampisilin, vankomisin, kloramfenikol'e duyarlı olduğu gösterilmiş ve bunların %40'ının bazı antibiyotiklere dirençli olduğu saptanmıştır. PFGE analizi 28 *Lactobacillus* suşunun 21 pulsetipine ayrıldığını ve A-B-C-D-E-F-G pulsetiplerindeki suşların

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%100 benzer olduğunu göstermiştir. Sonuç olarak, *Lactobacillus* türlerinin antibiyotik duyarlılıkları konusunda daha kapsamlı araştırmaların yapılması gerekmektedir.

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INTRODUCTION

Probiotics are live microorganisms that confer a health benefit on the host, as defined by the Food and Agriculture Organization of the United Nations and the World Health Organization. At the beginning of the 20th century, Ilja Metchnikoff reported that the longevity of Bulgarians is due to consumption of fermented milk products. Probiotic bacteria have been used as a health-promoting factor for a very long time (Zawistowska-Rojek & Tyski, 2018). Probiotics contribute to gastrointestinal and urogenital problems, allergic diseases and more generally, to improve the function of the digestive system and support the immune system (Villavicencio et al., 2018).

Probiotic properties have been seen in many genera of bacteria and fungi, but most used probiotics belong to the species of *Lactobacillus* and *Bifidobacterium*. Also, other bacteria genera, like *Streptococcus*, *Enterococcus*, and *Bacillus*, as well as members of the yeast genus *Saccharomyces* can have probiotic properties. The most common probiotic species contain: *Lactobacillus acidophilus*, *Lactobacillus johnsonii*, *Lactobacillus gasseri*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium bifidum* and *Bifidobacterium infantis* (Zawistowska-Rojek & Tyski, 2018). *Lactobacillus* strains are important members of the human and animal microbiomes, and are found in a variety of food products (Zhang et al., 2018). *Lactobacillus* contains 51 species among them the species *L. helveticus*, *L. kefiranofaciens*, *L. delbrueckii* and *L. kefiri*, which are commonly found in fermented milk (Georgalaki et al., 2021). Probiotics are widely available in raw's milk and conventional dairy products. Fresh or fermented cow's and goat's milk is consumed in different regions of the world. The presence of high counts of probiotic bacteria in both cow's and goat's milk important a source for public health (Reuben et al., 2020). Probiotic bacteria are becoming more and more resistant to clinically significant antibiotics, and this is linked to their improper usage in farm animals (Jaimee & Halami, 2016). Antibiotic resistance genes that can be passed on to pathogenic bacteria can be transferred by probiotic bacteria (Danielsen & Wind, 2003). It is quite concerning that lactic acid bacteria, a healthy component of the microflora, are developing antibiotic resistance (Jaimee & Halami, 2016). It is very important to determine the antibiotic susceptibility of probiotic bacteria (Danielsen & Wind, 2003).

The aim of the study is to identify different species of *Lactobacillus* in goat's and cow's milk collected from various farms in the province of Adana, as well as to investigate phylogenetic relationships and examine the antibiotic profiles of these strains.

MATERIALS and METHODS

In the study, milk of 10 cow's and 5 goat's, which were offered for daily consumption between 10.01.2022 and 07.02.2022, was taken from some farms in Adana province. Milk samples were taken into sterile capped plastic tubes kept in an ice box and transported to the laboratory. To identify the *Lactobacillus*, 10 mL and 40 mL of each sample were inoculated into de Man, Rogosa, and Sharpe (MRS) broth and incubated at 37°C under anaerobic conditions. All tubes with turbidity were then incubated on MRS agar plates and incubated for 24 to 72 hours at 37°C under anaerobic conditions. All strains were tested for Gram staining, catalase test, and coagulase reaction. The Crystal (BD BBL CRYSTAL ANR) technique was used to phenotypically identify several colonies thought to be *Lactobacillus* (Kızılyıldırım & Köksal, 2021).

Antibiotic Susceptibility

The susceptibility of *Lactobacillus* to antibiotics was evaluated by the disc diffusion test according to the criteria recommended by the National Committee for Clinical Laboratory Standards (Xu et al., 2012). The antibiotic susceptibility of the strains was assessed using antibiotics such as ampicillin (10 µg), vancomycin (30 µg), tetracycline (30 µg), erythromycin (15 µg), gentamicin (10 µg), and chloramphenicol (30 µg).

Phylogenetic Relationship of *Lactobacillus* strains

The phylogenetic relationships of *Lactobacillus* strains were done as previously described. Using the CHEF-DRII system (Bio-Rad Laboratories), DNA fragments were electrophoresed in 0.5 x TBE buffer for 22 hours at 14°C (Brennan et al.,2002; Xu et al., 2012). The GelComparII software program (version 4.0 Applied Maths, Sint-Martens-Latem, Belgium) was used to examine the PFGE data. The relationship between the strains were determined according to the "Dice"

similarity coefficient depending on the bands. The isolates with 100% similarity in band profiles were evaluated in the same cluster (Xu et al., 2012).

RESULTS and DISCUSSION

In the study, a total of 18 *Lactobacillus* strains were recovered from 10 different cow's milk, including seven *L. casei* (38.9%), five *L. rhamnosus* (27.8%), four *L. acidophilus* (22.2%), and two *L. fermentum* (11.1%). One of the milk samples (R1) included a combination of *L. acidophilus*, *L. casei*, and *L. rhamnosus*. Three milk samples (R6-R8-R9) had only one strain of *Lactobacillus*. Six samples of milk (R2-R3-R4-R5-R7-R10) included two different species of *Lactobacillus* (Table 1).

Table 1. *Lactobacillus* species isolated in cow's milk
Çizelge 1. İnek sütünden izole edilen Lactobacillus türleri

Number	Strain no	*Lactobacillus*	Number	Strain no	*Lactobacillus*
1	R1.1	*L. acidophilus*	10	R5.1	*L. acidophilus*
2	R1.2	*L. casei*	11	R5.2	*L. casei*
3	R1.3	*L. rhamnosus*	12	R6	*L. rhamnosus*
4	R2.1	*L. fermentum*	13	R7.1	*L. rhamnosus*
5	R2.2	*L. rhamnosus*	14	R7.2	*L. casei*
6	R3.1	*L. acidophilus*	15	R8	*L. fermentum*
7	R3.2	*L. casei*	16	R9	*L. casei*
8	R4.1	*L. casei*	17	R10.1	*L. casei*
9	R4.2	*L. acidophilus*	18	R10.2	*L. rhamnosus*

When the antibiotic susceptibility profiles of the *Lactobacillus* strains isolated from cow's milk were examined, it was found that seven strains (38.9%) were resistant to antibiotics and 11 strains (61.1%) were sensitive. Of the resistant *Lactobacillus* strains, six were resistant to tetracycline, two to gentamicin, one to ampicillin, and one to erythromycin. Vancomycin and chloramphenicol sensitivity were discovered in all strains. One of the *L. rhamnosus* strains showed resistance to both tetracycline and gentamicin. One strain of *L. casei* showed multiple resistance to ampicillin, tetracycline, and gentamicin. Among the *L. rhamnosus* strains, gentamicin resistance was found in one strain (20%) and tetracycline resistance in four (80%). Only one of the *L. fermentum* strains had tetracycline resistance (50%). In *L. casei* strains, one strain showed resistance to tetracycline (14.2%), gentamicin (14.2%), and ampicillin (14.2%), while one strain showed resistance to erythromycin (14.2%). All *L. acidophilus* strains have been demonstrated to be antibiotic susceptible (Table 2).

Table 2. Antibiotic susceptibility profiles of *Lactobacillus* strains isolated from cow's milk
Çizelge 2. İnek sütünden izole edilen Lactobacillus suşlarının antibiyotik duyarlılık profilleri

No	Strain no	*Lactobacillus*	*Amp	*Van	*Gen	*Ery	*Chl	*Tet
1	R1.1	*L. acidophilus*	S	S	S	S	S	S
2	R1.2	*L. casei*	S	S	S	S	S	S
3	R1.3	*L. rhamnosus*	S	S	S	S	S	R
4	R2.1	*L. fermentum*	S	S	S	S	S	R
5	R2.2	*L. rhamnosus*	S	S	S	S	S	R
6	R3.1	*L. acidophilus*	S	S	S	S	S	S
7	R3.2	*L. casei*	S	S	S	S	S	S
8	R4.1	*L. casei*	S	S	S	S	S	S
9	R4.2	*L. acidophilus*	S	S	S	S	S	S
10	R5.1	*L. acidophilus*	S	S	S	S	S	S
11	R5.2	*L. casei*	S	S	S	S	S	S
12	R6	*L. rhamnosus*	S	S	R	S	S	R
13	R7.1	*L. rhamnosus*	S	S	S	S	S	S
14	R7.2	*L. casei*	S	S	S	S	S	S
15	R8	*L. fermentum*	S	S	S	S	S	S
16	R9	*L. casei*	R	S	R	S	S	R
17	R10.1	*L. casei*	S	S	S	R	S	S
18	R10.2	*L. rhamnosus*	S	S	S	S	S	R

*Amp: Ampicillin, Van: Vancomycin, Gen: Gentamicin, Ery: Erythromycin, Chl: Chloramphenicol, Tet: Tetracycline.

Gad et al. (2014) isolated a total of 152 *Lactobacillus* spp. from 180 pharmaceutical and dairy samples. *Lactobacillus* isolates have shown the highest penicillin resistance. Almost a high percentage of

Lactobacillus isolates showed moderate resistance to cephalexin and a low percentage were resistant to cefoperazone (Gad et al., 2014). Hleba et al. (2021) reported that *Lactobacilli* isolated from milk and dairy products were resistant to erythromycin (21.4%), ampicillin (30.9%), and tetracycline (14.2%), but completely sensitive to gentamicin (Hleba et al., 2012). Bargezar et al. (2021) isolated *L. brevis*, *L. acidophilus*, *L. plantarum*, and *L. casei* species from six different raw milk cheeses. It was reported that 57% of the strains were resistant to kanamycin and 28% were resistant to tetracycline, with no resistance to chloramphenicol or erythromycin found (Barzegar et al., 2021).

The differences in *Lactobacillus* species and numbers in the studies are related to both the number of samples and the methods used in identification. In this study, the phenotypic method was used for the identification of *Lactobacilli*. However, if it was identified by the genotypic method, the number and type of *Lactobacilli* could change. On the other hand, antibiotic profiles in studies may differ depending on the antibiotic groups used and the number of strains.

A total of ten *Lactobacillus* strains were found in five different goat's milk, including five *L. casei* (50%), three *L. rhamnosus* (30%), and two *L. fermentum* (2%) strains. Each goat's milk sample (G1-G2-G3-G4-G5) included two distinct strains of *Lactobacillus* (Table 3).

Table 3. *Lactobacillus* species isolated in goat's milk
Çizelge 3. Keçi sütünden izole edilen Lactobacillus türleri

Number	Strain no	*Lactobacillus*
1	G1.1	*L. casei*
2	G1.2	*L. rhamnosus*
3	G2.1	*L. fermentum*
4	G2.2	*L. casei*
5	G3.1	*L. rhamnosus*
6	G3.2	*L. casei*
7	G4.1	*L. casei*
8	G4.2	*L. fermentum*
9	G5.1	*L. casei*
10	G5.2	*L. rhamnosus*

It was shown that six strains (60%) were sensitive and four strains (40%) were resistant when the antibiotic susceptibility profiles of the *Lactobacillus* strains isolated from goat's milk were examined. Two of the *Lactobacillus* strains had tetracycline resistance, one had gentamicin resistance, and two had erythromycin resistance. All strains were found to be sensitive to ampicillin, vancomycin and chloramphenicol. One of the *L. rhamnosus* strains demonstrated both erythromycin and gentamicin resistance. Two of the *L. casei* isolates showed a 50% tetracycline resistance. Two of the *L. rhamnosus* strains had erythromycin resistance (66.7%), whereas one had gentamicin resistance (33.3%). All strains were found to be sensitive to ampicillin, vancomycin, and chloramphenicol (Table 4).

Table 4. Antibiotic susceptibility profiles of *Lactobacillus* strains isolated from goat's milk
Çizelge 4. Keçi sütünden izole edilen Lactobacillus suşlarının antibiyotik duyarlılık profilleri

No	Strain no	*Lactobacillus*	*Amp	*Van	*Gen	*Ery	*Chl	*Tet
1	G1.1	*L. casei*	S	S	S	S	S	R
2	G1.2	*L. rhamnosus*	S	S	R	R	S	S
3	G2.1	*L. fermentum*	S	S	S	S	S	S
4	G2.2	*L. casei*	S	S	S	S	S	R
5	G3.1	*L. rhamnosus*	S	S	S	R	S	S
6	G3.2	*L. casei*	S	S	S	S	S	S
7	G4.1	*L. casei*	S	S	S	S	S	S
8	G4.2	*L. fermentum*	S	S	S	S	S	S
9	G5.1	*L. casei*	S	S	S	S	S	S
10	G5.2	*L. rhamnosus*	S	S	S	S	S	S

*Amp: Ampicillin, Van: Vancomycin, Gen: Gentamicin, Ery: Erythromycin, Chl: Chloramphenicol, Tet: Tetracycline.

The most prevalent *Lactobacillus* species found in goat's milk are *L. plantarum*, *L. rhamnosus*, *L. casei*, and *L. paracasei*. The high-potential *Lactobacillus* selection derived from goat's milk is industrially significant (Marroki et al., 2011).

Marroki et al. (2014) identified 19 *Lactobacillus* strains from goat's milk, including *L. plantarum* (13), *L. pentosus* (3), *L. rhamnosus* (2), and *L. fermentum*. *Lactobacillus* strains were discovered to be penicillin and erythromycin sensitive. All of the strains were resistant to vancomycin. It has been reported that resistance rates to other antibiotics differ according to *Lactobacillus* strains (Marroki & Bousmaha-Marroki, 2014).

In another investigation, antibiotic susceptibilities of 61 *Lactobacillus* strains (*L. plantarum* (28), *L. pentosus* (22), *L. fermentum* (6) and *L. rhamnosus* (5)) isolated from 14 raw goat's milk samples were tested. Most of the strains were more sensitive to β-lactam group antibiotics (penicillin G (52%), ampicillin (82%) and amoxicillin (80%)). It was also susceptible to

cefotaxime (39%) and imipenem (56%). In addition, high susceptibility to protein synthesis inhibitors such as erythromycin (48%), tetracycline (49%), chloramphenicol (80%), and fusidic acid (26%) were observed. All isolates were resistant to oxacillin, ceftazidine, ceftriaxone, vancomycin, and trimitoprim-sulfamide (Bousmaha-Marroki & Marroki, 2015). Marroki et al. (2011) reported that *Lactobacillus* strains isolated from goa't milk were sensitive to tetracycline, erythromycin and resistant to vancomycin, kanamycin and gentamicin (Marroki et al., 2011). The results of this study are similar to other studies on goat's milk in terms of antibiotic profiles and *Lactobacillus* species. However, further research is needed on the antibiotic profiles of the strains of *Lactobacillus* isolated from goat's milk samples.

It is believed that the primary means of transmission of bacteria resistant to antibiotics across populations of animals and people is through the food chain (Erginkaya et al., 2018). In particular, probiotic organisms are thought to transmit antibiotic resistance genes to pathogenic bacteria. It should be remembered that some *Lactobacillus* species can be resistant to antibiotics and can help other microorganisms acquire antibiotic resistance genes (Wang et al., 2019). In this regard, the antibiotic susceptibilities of probiotic microorganisms in foods should be evaluated, and more extensive research is necessary.

In the evaluation of clonal relationships of *Lactobacillus* strains by the PFGE method, it was observed that 28 strains were divided into 21 pulsetypes. The two-membered A-B-C-D-E-F-G pulsetypes *Lactobacillus* strains were found to be similar (100%). The other 14 strains were separated into unrelated single-membered pulsetypes (Figure 1). It was observed that *L. rha*mnosus isolates in the A pulse type were resistant to tetracycline, *L. rhamnosus* (R6) strains in the E pulsetype were resistant to tetracycline and gentamicin, and *L. rhamnosus* (R10.2) was found to be resistant to tetracycline. Additionally, tetracycline resistance was detected in *L. casei* strains of the G pulsetype, as well as gentamicin and erythromycin resistance in *L. rhamnosus* (G1.2) *and L.* rhamnosus (G3.1) strains of the F pulsetype. Tetracycline resistance was also found in *L. rhamnosus* strains of the F pulsetype.

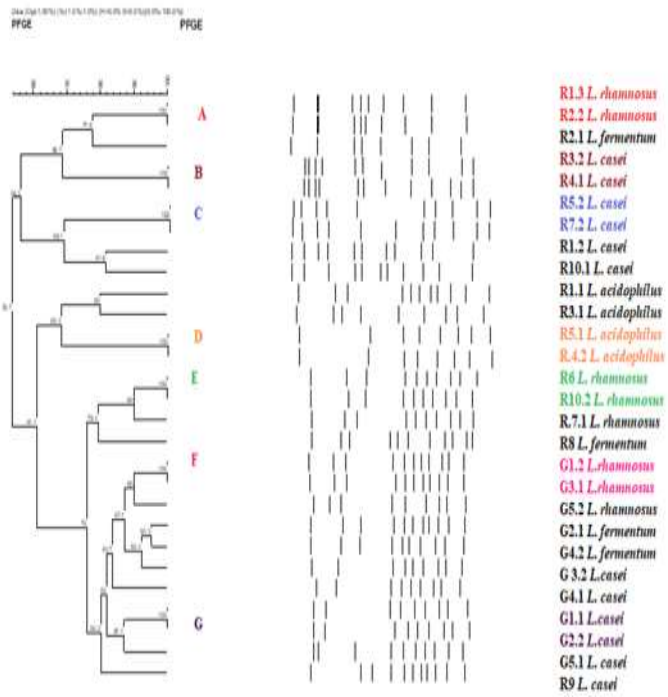

Figure1. Phylogenetic relationship of *Lactobacillus* strains
Şekil 1. Laktobasil suşlarının filogenetik ilişkisi

Similar to this study, Xu et al. (2012) found that the PFGE analysis separated 33 *Lactobacillus* strains into 17 pulsetypes. According to their findings, different *Lactobacillus* strains exhibited the same PFGE patterns and likely descended from a common ancestor of these strains. They found that all antibiotic resistance patterns of each strain were similar in A, C, F, J, K, and M pulsetypes, and some strains with

different pulsetypes exhibited the same antibiotic resistance spectrum (Xu et al., 2012).

CONCLUSION

In conclusion, we strongly believe that studies on the investigation of *Lactobacillus* species and elucidation of their antibiotic susceptibility profiles in foods such as milk and dairy products should continue. It should be encouraged to consciously use antibiotics in animal illnesses in order to prevent antibiotic resistance.

Author's Contributions

The authors declare that they have contributed equally to the article.

Statement of Conflict of Interest

Authors have declared no conflict of interest.

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