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Cell-free supernatants of lactobacilli inhibit methicilin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus* and carbapenem-resistant *Klebsiella* strains

Laktobasillerden elde edilen hücresiz süzüntülerin metisiline dirençli Staphylococcus aureus, vankomisine dirençli Enterococcus ve karbapeneme dirençli Klebsiella suşlarını inhibisyonu

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ABSTRACT

Aim: Antibiotic resistance is a major health problem. Recently, probiotics are used in the field of alternative/supportive medicine. Thus, in this study, we aimed to evaluate the *in vitro* inhibitory effects of four different cell-free supernatants (CFSs) of lactobacilli species (*L. fermentum, L. plantarum*, *L. acidophilus* and *L. rhamnosus*) on clinically isolated Methicilin-resistant *Staphylococcus aureus* (MRSA) strains, Vancomycin-resistant *Enterococcus* (VRE) strains, and Carbapenem-resistant *Klebsiella* (CRK) strains.

Materials and Methods: Lactobacillus strains were grown in de Man Rogosa Sharpe broth; after filtration, CFSs were diluted to obtain 25, 50, and 100% concentrations. Pathogen bacteria were grown in tryptic soy broth with and without CFSs in a micro-plate. The bacterial growths were measured using spectrophotometric method after four hours of incubation at 37°C. One-way ANOVA followed by Dunnett's multiple comparisons and Kruskal-Wallis test were used for statistical analyses.

Results: All tested CFSs at all concentrations were found to inhibit growth of MRSA, VRE, CRK strains; the results were found statistically significant ($p<0.0001$). At 50% concentrations, all CFSs were found to be most effective on MRSA growth. The CFSs of *L.fermentum, L.acidophilus* and *L.plantarum* were found to be most inhibitory at 50% concentration on VRE growth. 50% diluted CFSs of *L.fermentum* and *L.plantarum* were found to be effective on growth of CRK. All results were found statistically significant ($p<0.0001$).

Conclusion: In our study, our results support that CFSs of lactobacilli strains inhibit growth of multi-drug resistant bacteria. Their inhibitory effects were dependent on microorganisms and CFS concentrations.

Keywords: Lactobacilli, Cell free supernatant, MRSA, VRE, CRK, growth inhibition.

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ÖZ

Amaç: *Antibiyotik direnci büyük bir sağlık sorunudur. Son yıllarda, probiyotikler alternatif/destekleyici tıp alanında sık kullanılmaktadır. Bu nedenle, çalışmamızda, dört farklı lactobacillus türünün (L. fermentum, L. plantarum, L. acidophilus ve L. rhamnosus) hücresiz süzüntülerinin (cell-free supernatant, CFS) etken olarak izole edilen metisiline dirençli Staphylococcus aureus (MRSA), vankomisine dirençli Enterococcus (VRE) ve karbapeneme dirençli Klebsiella (KRK) suşlarının üremeleri üzerine inhibitör etkilerini in vitro saptamayı amaçladık.*

Gereç ve Yöntem: *Lactobacillus suşları de Man Rogosa Sharpe sıvı besiyerinde üretilmiştir; hücresiz süzüntüler %25, 50 ve 100'lük konsantrasyonları elde etmek için sulandırılmıştır. Patojen bakteriler mikroplaklarda, farklı konsantrasyonlarda CFS içeren ve içermeyen triptik soy buyyonda üretilmişlerdir. Üremeler, 37°C'deki dört saatlik inkübasyon sonunda spektrofotometrik yöntemle ölçülmüştür. İstatistiksel analizler, one-way ANOVA kullanılarak, Dunnett'in çoklu karşılaştırma testi ve Kruskal-Wallis test ile gerçekleştirilmiştir.*

Bulgular: *İncelenen tüm CFS konsantrasyonlarının MRSA, VRE ve KRK suşlarının üremelerini inhibe ettiği saptanmış; sonuçlar istatistiksel olarak anlamlı bulunmuştur ($p<0.0001$). Tüm CFS'lerin %50'lik konsantrasyonlarının MRSA suşlarının üremesine en etkili konsantrasyon olduğu belirlenmiştir. L.acidophilus, L.fermentum ve L.plantarum CFS'lerinin %50'lik konsantrasyonlarının VRE suşlarının üremesine en etkili konsantrasyon olduğu bulunmuştur. L.fermentum ve L.plantarum CFS'lerinin %50'lik konsantrasyonlarının, KRK üremesini en etkili biçimde inhibe eden konsantrasyon olduğu saptanmıştır. Tüm sonuçlar istatistiksel olarak anlamlı bulunmuştur ($p<0.0001$).*

Sonuç: *Çalışmamızda sonuçlarımız denenen tüm laktobasil CFS'lerinin çoğul antibiyotik dirençli bakterilerin üremesini inhibe ettiğini desteklemektedir. Bu inhibitör etkileri, mikroorganizmalara ve CFS'lerinin konsantrasyonlarına bağlı olarak değişkendir.*

Anahtar Sözcükler: *Lactobacillus, hücresiz süzüntü, MRSA, VRE, KRK, üreme inhibisyonu.*

INTRODUCTION

Multi-drug resistance is a very big problem all over the world leading to treatment failures of nosocomial and community-acquired infections and responsible for high morbidity-, mortality rates, increasing of health charges. Resistance to various beta-lactam antibiotics generated by different mechanisms is prevalent in Gram-negative and Gram-positive bacteria (1, 2). In the last few years, it has been reported that the rates of colistin resistance in Gram-negative bacteria are increasing (3, 4). Besides, multi-drug resistant bacteria isolated from nosocomial infections such as vancomycin-resistant Enterococcus (VRE) strains and methicilin resistant *Staphylococcus aureus* (MRSA) strains become a concerning problem after horizontally transferring of vancomycin resistance from VRE to MRSA strains (5, 6).

In the last few decades, some alternative treatment and/or preventive strategies were investigated (1, 7, 8). Some previous studies suggested that probiotics can be used both for the protection from infections and as supplementary drugs for the treatment of infections especially caused by multi-drug resistant pathogens (9-15). Probiotics are characterized as living microorganisms that provide to promote host health (16). Lactobacillus species are known as the major probiotic microorganisms that have protective roles against pathogens by competing, secretion some antimicrobial substances against them, and supporting the host immune system (17-20).

In our study, it was aimed to investigate the effects of cell-free supernatants (CFSs) obtained from different lactobacilli (*Lactobacillus fermentum* ATCC 9338, *Lactobacillus plantarum* ATCC 14917, *Lactobacillus acidophilus* ATCC 314 and *Lactobacillus rhamnosus* ATCC 53103) on the growth of clinically isolated Methicilin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant Enteroccoccus (VRE) and Carbapenem-resistant Klebsiella strains (CRK).

MATERIALS and METHODS

Bacteria

In the present study, 30 MRSA strains, 30 CRK strains, 30 VRE strains were isolated from patients administered to Sağlık Bilimleri University, Faculty of Medicine, Haydarpaşa Numune Hospital, Istanbul Yeni Yüzyıl University Faculty of Medicine, Gaziosmanpaşa Hospital and Istanbul University, Istanbul Faculty of Medicine, respectively. MRSA and CRK strains were isolated from various clinical samples

(Table-1) and VRE strains were isolated from rectal swab samples.

The antimicrobial susceptibilities were determined by the disk diffusion method and automatized systems (VITEK-2) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines.

L. rhamnosus ATCC 53103, *L. fermentum* ATCC 9338, *L. acidophilus* ATCC 314 and *L. plantarum* ATCC 14917 were investigated in the present study. These particular strains were chosen according to their widely usage in various products such as gums, fruit drinks, and medical tablets which are mainly available in both markets and pharmacies (21, 22).

Before the experiments, we stored all strains at −80 °C.

Table-1. MRSA and CRK strains isolated from various clinical samples

Sample	MRSA	CRK
Blood culture	11	14
Ulcer swab	7	-
Tracheal aspiration	7	3
Sputum	1	1
Tissue biopsy	2	1
Abscesses / free abdominal fluid	1	2
Urine	1	9

Media and culture conditions

For the experiments, MRSA, VRE, and CRK strains were cultured in Tryptic Soy Broth (TSB) at 37 °C in aerobic conditions for 24 hours. For isolation of lactobacilli, de Man Rogosa Sharpe (MRS) broth was used, bacteria incubated under anaerobic conditions at 37°C for 48 hours.

The overnight cultures of lactobacilli were centrifuged at 4000 rpm for 30 minutes at 4 °C then the supernatants were filtered by using a 0.2 µm filter (23). In the experiments, diluted (25% and 50%) and non-diluted cell-free supernatants (CFSs) from each of the lactobacillus strains were used to determine the most effective inhibitory concentration.

The detection of growth alterations of MRSA, CRK, and VRE strains in the presence of cell-free Lactobacilli supernatants

The initial concentrations of bacteria were 10^7 CFU/mL. Bacteria were cultured into TSB alone (as control) and TSB added different CFSs of lactobacilli (80 µl TSB+ 20 µl bacteria and 100 µl CFS). We incubated all strains at 37 °C for 4 hours and the growth was measured according to their absorbance values at 600 nm. The differences in optic density values obtained from control and experimental conditions were compared. All experiments were repeated twice.

Statistical analysis

Statistical analysis was determined using one-way ANOVA followed by Dunnett's multiple comparisons test and Kruskal-Wallis test. Multiple comparisons were made at a level of $P<0.05$.

RESULTS

In the present study, we found that all CFSs at all concentrations inhibited MRSA growth. (Figure-1a-1c) and the difference was statistically significant ($p<0.0001$). All 50% diluted-CFSs have been found most effective on MRSA strains (Figure-1b).

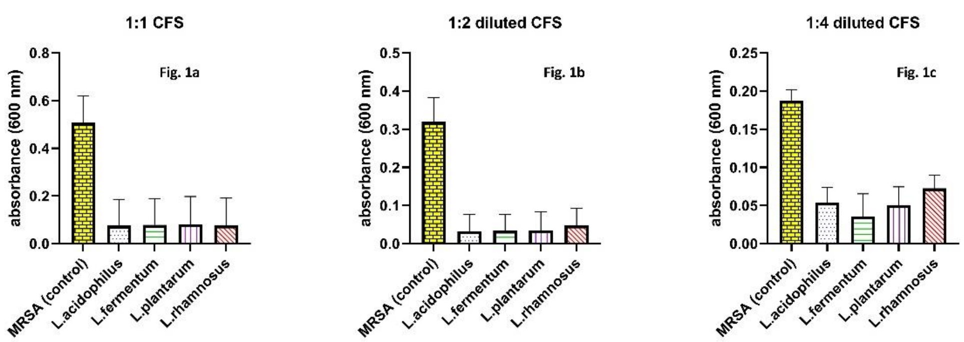

Figure-1. The effects of various lactobacilli CFSs on the growth of MRSA strains. The significance of growth inhibition was determined by comparing growth under control conditions (only MRSA growth, without CFS). The statistical analysis was done using one-way ANOVA followed by Dunnett's multiple comparisons test. The significant differences were at $p <0.0001$ level.

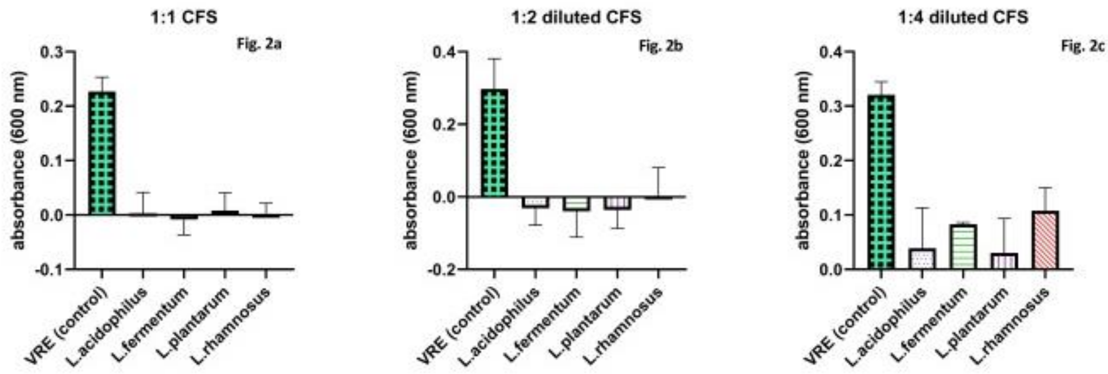

Figure-2. The effects of various lactobacilli CFSs on the growth of VRE strains. The significance of growth inhibition was determined by comparing growth under control conditions (only VRE growth, without CFS). The statistical analysis was done using one-way ANOVA followed by Dunnett's multiple comparisons test. The significant differences were at p <0.0001 level.

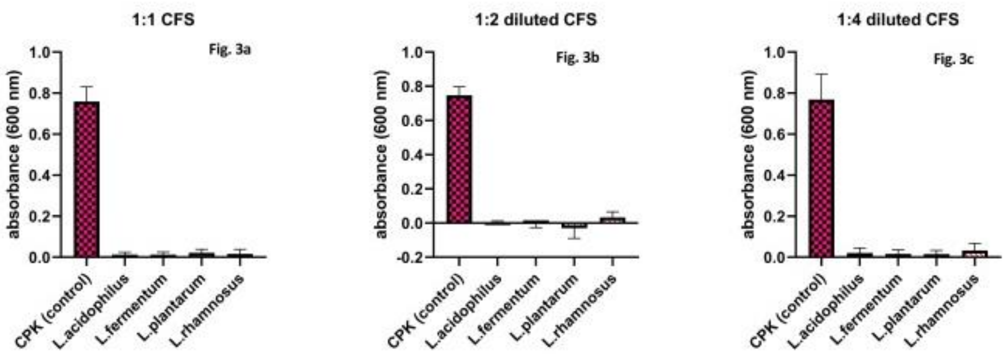

Figure-3. The effects of various different lactobacilli CFSs on the growth of CRK strains. The significance of growth inhibition was determined by comparing of growth under control conditions (only CRK growth, without CFS). The statistical analysis was done using one-way ANOVA followed by Kruskal-Wallis test. Significant difference at p<0.0001 level.

All tested CFSs and their concentrations were also found to decrease the growth of VRE strains, and these results were statistically significant (p<0.0001) when compared to control (Figure-2a-2c). *L. fermentum's* non-diluted-CFS has been found as the most effective antibacterial compound on VRE strains (Figure-2a). All CFSs, except CFS of *L. rhamnosus,* at 50% concentration were found to the most inhibitory effect on VRE growth than other concentrations (Figure-2b).

All tested CFSs at all concentrations were found to reduce the growth of CRK strains (Figure-3a-3c). The growth reduction was found to be statistically significant (p<0.0001) for all CFSs. 50% diluted-CFSs of *L. fermentum* and *L. plantarum* have been found as the most effective antibacterial compounds on CRK strains (Figure-3b).

DISCUSSION

It is well known that antibiotic resistance is a major and growing problem in the treatment of infectious diseases, thus alternative strategies are investigated. In our study, we evaluated the *in-vitro* inhibitory effects of various CFSs obtained from lactobacillus species on clinically multidrug-resistant MRSA, VRE, and CRK strains which are known as concerning resistance patterns all around the world.

It has been reported that lactobacilli can increase the phagocytosis of macrophages and prevent the colonization of pathogens by competing with other microorganisms and/or by providing hydrogen peroxide, lactic and organic acids, bacteriocins and bio-surfactants to modulate environmental conditions (24-28). In accordance with these effects, it is well known that lactobacilli are the most investigated probiotics; their preventive and supportive effects against pathogens have been detected in experimental studies and clinical trials (9-15, 29-32). Previous studies have proven that lactobacilli and their CFSs have effects on growth, adhesion, invasion, biofilm formation, oxidative damage, bacterial cell membrane permeability and gene expression of microorganisms (24, 33-35). Moreover, some *in vivo* studies also supported these inhibitory effects of lactobacilli (15, 32).

In recent years, attention is directed to the inhibitory effects of probiotics on multi-drug resistant, problematical bacteria. Onbas et al. (2019) reported that CFS of *L. plantarum* strain inhibited the growth of different MRSA strains isolated from skin infections and they suggested that, a product of *L. plantarum* represents a proper strategy for bio-control against wound infections (32). In Malaysia, CFSs of six different *L. acidophilus* strains isolated from honey samples were shown to inhibit the growth of multi-drug resistant bacteria (*S. aureus*, *S. epidermidis* and *B. subtilis* strains) in a pH-dependent manner (9). Therefore, acidic pH of CFSs was found to be important for their antibacterial effects. Similarly, Bhola and Bhadekar (2019) showed that the mixed cell lysate of different lactobacilli strains inhibited the growth of multi-drug resistant *Staphylococcus* isolates at a rate of 85% (10). We found that CFSs of all lactobacilli strains we tested have inhibitory effect on growth of MRSA strains. Particularly, we found that the most effective inhibitory concentration was 50% for all CFSs. Thus, it seems that lactobacilli products have an important potential for the prevention of *S. aureus* growth.

There are also many studies reporting effects of CFSs obtained from various lactobacilli on problematical resistant Gram-positive bacteria other than MRSA (36-38). Naderi et al. (2014) indicated that *L. acidophilus*, *L. casei* and *L. rhamnosus* have no antagonistic effect against multi-drug resistant *Enterococcus* species which were isolated from urinary tract infections (14). In contrast to these results, Sun et al. (2009) concluded that *L. rhamnosus* decreased the growth of *E. faecalis* (39). Similarly, Thanh et al. (2010) have shown that the metabolites of different *L. plantarum* strains' combinations inhibited the growth of *S.Typhimurium, E.coli, L. monocytogenes* and VRE (40). This result is consistent with those obtained by Mahdi et al. (2012), CFSs of *L. fermentum* obtained from vaginal swabs shown to have significant antibacterial activity on *E. faecalis* and *E. faecium* strains (41). In line with these studies, our findings indicated that all lactobacilli CFSs at all concentrations had an antibacterial effect on VRE strains. Moreover, when we used the non-diluted CFSs, we found that *L. fermentum* had the most antagonist effect than others. It is worthy to note that, CFSs of *L. acidophilus, L. fermentum* and *L. plantarum,* at 50% concentrations were found to be most effective.

Similar findings were reported in studies on growth of gram-negative bacteria (11, 12, 14, 22, 25, 34, 35, 42). Chen et al. (2019) have shown that *L. paracasei*, *L. plantarum* and *L. rhamnosus* strains inhibit the growth of carbapenem-resistant *Escherichia coli* and *Klebsiella pneumoniae* strains (11). Fedorova et al. (2017) have suggested that *L. rhamnosus*, *L. reuteri* and *Lactobacillus helveticus* had significant antagonistic activity against multiple antibiotic-resistant *Klebsiella pneumoniae* strains (43). The study conducted by El Mokhtar et al. (2020) has shown that *L. acidophilus*'s CFS reduced the growth of ESBL producing *K. pneumoniae* and *P. aeruginosa* strains in a time-dependent manner. They also concluded that usage of CFS may become an effective strategy to overcome infections caused by these resistant bacteria (12). Raras et al. (2019) showed that the cell viability rate of *K. pneumoniae* strain isolated from a patient with severe pneumonia decreased to 29.77% in the presence of CFS of lactobacilli isolated from kefir (44). On the contrary to these results, Naderi et al. (2013) reported that *L. acidophilus*, *L. casei*, and *L. rhamnosus* have no antagonistic effect against multi-drug resistant *K. pneumoniae* and *Enterobacter* species isolated from urinary tract infections. They also found that only the growth of *E. coli* strains was inhibited in the presence of CFSs which are defined as resistant to 8 or 9 different antibiotics. *L. casei* was reported as the most effective probiotic (14). Inconsistent with Naderi, Saud et al. (2020) detected that CFSs of lactobacilli strains isolated from milk, were shown to have no inhibitory effects on multi-drug resistant *Klebsiella* and *Shigella* species; however it was shown that *S. aureus, E. coli, Salmonella Paratyphi*, *Salmonella Typhi, Pseudomonas*, *Proteus, Acinetobacter* strains were inhibited in the presence of CFSs (45). Gumus et al. (2020) have shown that the growth and biofilm formation of clinically isolated

Uropathogenic *E.coli* (UPECs) were inhibited in the presence of *L. rhamnosus*, *L. fermentum*, *L. acidophilus* and *L. plantarum* CFSs (42). In the present study, we found that all CFSs and all concentrations have inhibitory effect on CRK. Furthermore, *L. fermentum* and *L. plantarum's* CFSs at 50% dilutions were found to be the most effective against CRK.

CONCLUSION

Consistent with other studies, our results have shown that the inhibitory effects of CFSs of lactobacilli were found to vary depending on lactobacilli species, pathogens and concentration (11, 42).

With regard to our results, it is obvious that CFSs of *L.acidophilus, L.fermentum, L.plantarum* and *L.rhamnosus* inhibited the growth of MRSA, VRE, and CRK strains most effectively at 50% concentration. In accordance with previous studies, we expect, these findings will contribute for the development of alternative and preventive strategies for infectious diseases.

Conflict of interest

There is no conflict of interest to disclose.

Acknowledgment

Ethical approval has been obtained from Istanbul Yeni Yüzyıl University The Research Ethics Committee. Meeting Date: 11.01.2021 No: 01-562.

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