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Cell-Free *Lactobacillus* sp Supernatants Modulate *Staphylococcus aureus* Growth, Adhesion and Invasion to Human Osteoblast (HOB) Cells

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Abstract
The increase of antibiotic resistance has become a problem. Probiotic bacteria play an important role in preventive/supportive medicine. Therefore, we examined the inhibitory effects of four different Lactobacillus species' (*L. acidophilus*-La, *L. plantarum*-Lp, *L. fermentum*-Lf and *L. rhamnosus*-Lr) cell-free supernatants (CFSs) on growth, adhesion, invasion, and biofilm formation of *Staphylococcus aureus* and effects of *S. aureus*, CFSs, and *S. aureus*-CFSs co-existence on human osteoblast (HOB) cell viability. Growth alterations were measured spectrophotometrically. Adhesive/invasive bacterial counts were detected by colony counting. Biofilm was evaluated using microtiter plate assay. The MTT assay was used for detection of HOB cell viability. The growth of MSSA significantly ($P<0.01$) decreased in the presence of two CFSs (Lf and Lr) ($P<0.01$); the growth of MRSA significantly ($P<0.05$) reduced in the presence of La CFSs. All tested CFSs were found to reduce adhesion and invasion of MSSA ($P<0.0001$). The adhesion of MRSA was enhanced ($P<0.0001$) in the presence of all CFSs except La and the invasion of MRSA was decreased ($P<0.01$) in the presence of Lr and Lf CFSs. All tested CFSs were shown to inhibit biofilm formation significantly ($P<0.0001$). The reduction of *S. aureus* infected HOB cell viability and exposed to all CFSs except Lr that was found to be significant ($P<0.0001$). The viability of HOB cell during co-incubation with MSSA and CFSs was shown to be decreased significantly. However co-existence of MRSA and CFSs did not alter HOB cell viability. These results suggested that lactobacilli as probiotics have low protective effects on MRSA-infected host cells.

Introduction

Probiotics are defined as living microorganisms which promote the host health. There are some studies showing that probiotics can be used not only for prevention of infections, but also as alternative therapeutics for the treatment of infections especially caused by multi-drug resistant pathogens [1–4]. Lactobacilli are one of the most effective probiotics, for preventing and control of oral, gastrointestinal, and urogenital infections [5–12]. There are several mechanisms for the beneficial effects of probiotics such as immunomodulation, inhibition of pathogen's adhesion/invasion/growth due to the ability of probiotic microorganisms' formation of many compounds with fermentative and/or antimicrobial activities, secretions of proteins which degrade carbohydrate receptors, and production of receptor analogs and biosurfactants to compete against pathogens for nutrient and adhesion sites [2, 13–16].

In the present study, we aimed to evaluate the possible inhibitory effects of cell-free supernatants (CFSs) obtained from various lactobacilli on growth, adhesion, and invasion ability of *S. aureus* in human osteoblast cell (HOB) culture and in vitro biofilm formation. In the meantime, we aimed to detect the effects of CFSs and *S. aureus* and co-existence of *S. aureus* + CFSs on HOB cell viability.

Materials and Methods

Bacteria, Media, and Cell-Free Supernatants

In the present study, two different *Staphylococcus aureus* strains (MSSA ATCC 25923 and MRSA ATTC 43300) and four Lactobacillus species (*Lactobacillus rhamnosus* ATCC

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53103-Lr, *Lactobacillus fermentum* ATCC 9338-Lf, *Lactobacillus acidophilus* ATCC 314-La, and *Lactobacillus plantarum* ATCC 14917-Lp) were used. Lactobacilli were chosen according to their wide usage in dairy products, fruit drinks, chewing gums, and tablets available on market [17, 18].

Lactobacillus strains were grown in de Man-Rogosa-Sharpe (MRS) broth (Conda, Spain) under anaerobic and *S. aureus* strains were grown in Tryptic Soy Agar (TSA) under aerobic conditions at 37 °C for 24 h. All strains were stored in − 80 °C.

The supernatants (CFSs) from lactobacilli were obtained by filtration (0.2 µm pore size) followed by the centrifugation of the overnight cultures of lactobacilli at 4000 rpm for 30 min at 4 °C [19].

Human Osteoblast (HOB) Cell Culture

Human Osteoblast (HOB) cells (406-05F, Sigma-Aldrich) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) (Sigma, 5546) containing 50 U/mL penicillin and 50 µg/mL streptomycin (Biological Industries, 03-031-1B), 1% 2 mM L-glutamin (Biological Industries, BI03-020-1B), and 10% fetal bovine serum (FBS) (Biowest, S1810-500). Cells were seeded in 96-well plates (1×10^4 seeding density per each well) for growth and methylthiazolyldiphenyltetrazolium bromide (MTT) assays or in 24-well plates (5×10^4 seeding density per each well) for invasion and adhesion assays and incubated overnight at 37 °C under 5% CO_2 to obtain a confluent monolayer (4×10^4 cells in 96-well plates and 2.4×10^5 cells in 24-well plates at confluency) cell culture.

Prior to infection with *S. aureus*, cells were washed twice with phosphate-buffered saline (PBS), fresh antimicrobial solution-free DMEM, and 20 µL (per each well/96-well plates)/50 µL (per each well/24-well plates) CFS were added. After the well plates were incubated for 1 h [20, 21], the cells were infected with overnight culture of *S. aureus* in TSB (10^6 CFU/mL bacteria in each well) for 3 h [22].

The cells were incubated for 3 h for growth [22],1 h for adhesion [22–24],3 h for invasion assays [23, 24], and 4 h for viability assay [25] at 37 °C in CO_2.

Bacterial Growth

Cells seeded in 96-well plates with/without CFS of each lactobacillus and infected with each *S. aureus* were incubated at 37 °C with 5% CO_2 for three hours. Bacterial growth was measured at 600 nm via spectrophotometric method. The effects of each CFS were determined by comparing absorbance results of growth in infected HOB cell culture with/without CFSs.

All analyses were tested in duplicate and each experiment was performed twice.

Bacterial Adhesion

Cells seeded in 24-well plates with/without CFS of each lactobacillus and infected with each *S. aureus* were incubated at 37 °C under 5% CO_2 for one hour. The wells were washed three times with PBS to remove non-adherent bacteria. To detect adhered bacteria, cell cultures were treated with 500 µl 0.025% Triton X-100 for 5 min at 37 °C in 5% CO_2 to detach and lyse the cell monolayer. Bacterial colonies were counted after the cell lysates were inoculated on TSA and incubated at 37 °C for 24 h. The effects of each CFSs were determined by comparing colony counts obtained from cell lysates of cell cultures with and without CFS [23, 24].

All samples were tested in duplicate and each experiment was performed twice.

Bacterial Invasion

Cells seeded in 24-well plates with/without CFS of each lactobacillus and infected with each *S. aureus* were incubated at 37 °C under 5% CO_2 for 3 h. Cells were washed with PBS and fresh medium containing 200 µg/mL gentamycin was added to kill extracellular bacteria. The plates were re-incubated at 37 °C for one hour in 5% CO_2. To remove extracellular bacteria, cells were washed three times with PBS and 500 µl 0.025% Triton X-100 was used to lyse the cells. Cell lysates were homogenized and inoculated on TSA and incubated at 37 °C for 24 h to detect invasive bacteria. The effects of each CFS were determined by comparing colony counts obtained from cell lysates of cell cultures with and without CFS [23, 24].

All samples were tested in duplicate and each experiment was performed twice.

Bacterial Biofilm Formation

For the detection of biofilm formations, microtiter plate assay was used.

Bacteria were cultured in TSB-glucose (1%v/v) at 37 °C for 24 h and diluted 1/50 in fresh TSB-glucose, yielding a final concentration of approximately 10^7 CFU/mL. Each well containing 20 µL from cultivated bacteria, 80 µL TSB, and 100 µL of each cell-free supernatant were incubated at 37 °C for 24 h. After incubation, supernatants were aspirated from wells gently and the wells were washed three times with 250 µL PBS to remove any unattached bacteria and air-dried. 200 µL of 99% methanol was added to each well to fixate for 15 min and aspiration was performed in each well. To detect biofilm mass, wells were stained with 200 µL 0.1% crystal violet (in water) for 5 min. Excess stain was gently rinsed

off with tap water, and the plates were air-dried. The stain was solubilized by adding 200 µL of 95% ethanol. Biofilm formations were determined by optical density measurement in a spectrophotometer at 450 nm. We interpreted our results, according to four categories as described previously by Christensen et al. (1985) [26].

We used *E. coli* ATCC 25,922 as positive control (a biofilm forming strain was used as an internal control) and TSB-glucose (without CFSs) as negative control.

The assay was repeated three times.

Cell Viability

Cells seeded in 96-well plates with/without CFS of each Lactobacillus are infected/non-infected with each *S. aureus* strains and incubated at 37 °C under 5% CO_2 for three hours. After the wells were washed three times with PBS to remove all residues including medium, bacteria, and CFSs, fresh culture media was added to each well. Cell viability was examined by methylthiazolyldiphenyltetrazolium bromide (MTT) assay. Stock solution of MTT (12 mM) (Neofrox 3580 MTT) was prepared as described by Mosmann (1983) and added to each well [25]. After incubation at 37 °C for 4 h, the media was removed from the wells and the remained formosan crystals in wells were dissolved in 70 µL of dimethyl sulfoxide (DMSO) for 10 min. Absorbance was measured at 540 nm. The effects of each *S. aureus* and CFSs seperatly on HOB cells and the effects of each *S. aureus* on HOB cells in the presence of *S. aureus* strains and CFSs together were determined by comparing absorbance results.

All samples were tested in duplicate and each experiment was performed twice.

Statistical Analysis

Statistical analysis was determined by using one-way ANOVA followed by Tukey's multiple comparisons test. All measurements were compared to control conditions. All results are presented as mean ± SD.

Multiple comparisons were made at a level of $P < 0.05$ and the significance values were indicated as *: $P < 0.05$, **: $P < 0.01$, and ***: $P < 0.001$ in the manuscript.

Results

Bacterial Growth

The growth of MSSA was statistically significantly ($P < 0.01$) decreased in the presence of Lf and Lr CFSs ($P < 0.01$), while the growth of MRSA was statistically significantly ($P < 0.05$) reduced in the presence of only La' CFSs (Fig. 1a, b).

Bacterial Adhesion

All tested CFSs were found to reduce the adhesion of MSSA, and these results were statistically significant ($P < 0.0001$) when compared with the result of the experiment without CFSs.

The adhesion of MRSA was enhanced in the presence all of CFSs except La, and these results were statistically significant ($P < 0.0001$) (Fig. 2a, b).

Bacterial Invasion

All tested CFSs were found to reduce the invasion of MSSA, the differences were found to be statistically significant ($P < 0.0001$) when compared with the result of the experiment without CFSs. However, the invasion of MRSA was decreased in the presence of Lr and Lf CFSs, and these results were statistically significant ($P < 0.01$) (Fig. 3a, b).

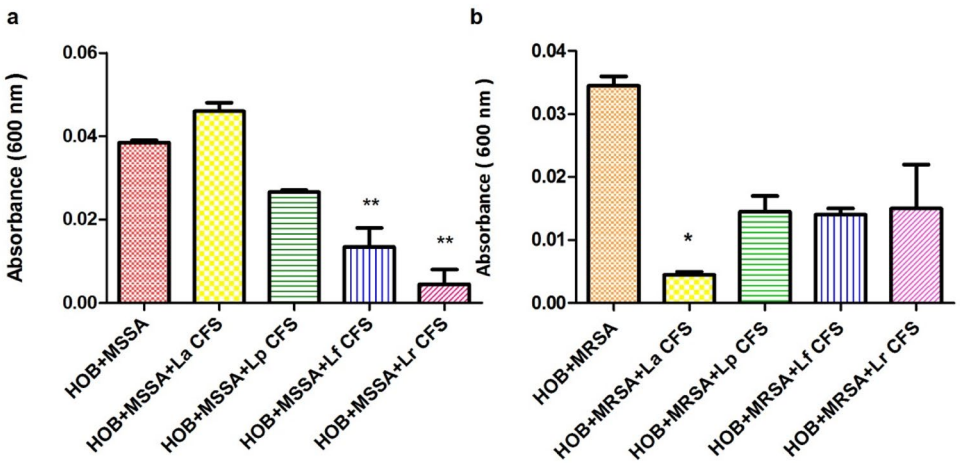

Fig. 1 Effects of different lactobacilli CFSs on growth of *S. aureus* strains. The alterations were determined by comparing with control (*S. aureus*-infected HOB cells). **a** Growth alterations of MSSA; **b** Growth alterations of MRSA. The growth of bacteria in the presence of HOB cells were examined using one-way ANOVA followed by Tukey's multiple comparisons test *,**: Significant at $P < 0.05$ level and $P < 0.01$ level values, respectively

Bacterial Biofilm Formation

All tested cell-free supernatants of lactobacilli were shown to inhibit biofilm formation of both *S. aureus* strains in 24 and 48 h significantly ($P < 0.0001$) (Fig. 4a, b).

The figure shows the average absorbance results of both 24 and 48 h assays.

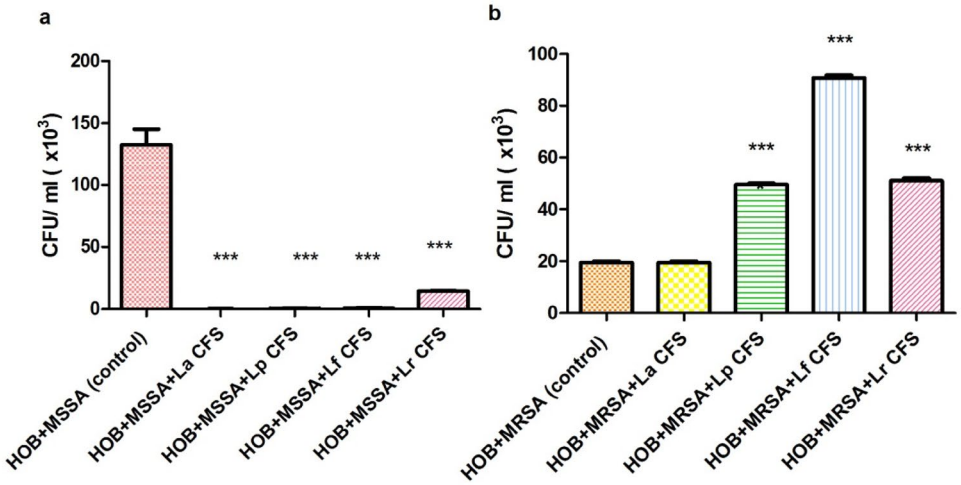

Fig. 2 Effects of different lactobacilli CFSs on adhesion of *S. aureus* strain. The alterations were determined by comparing with control (*S. aureus*-infected HOB cells). **a** Counts of adhered MSSA; **b** Counts of adhered MRSA. The adhesion of bacteria in the presence of HOB cells were examined using one-way ANOVA followed by Tukey's multiple comparisons test ***: Significant at $P < 0.0001$ level

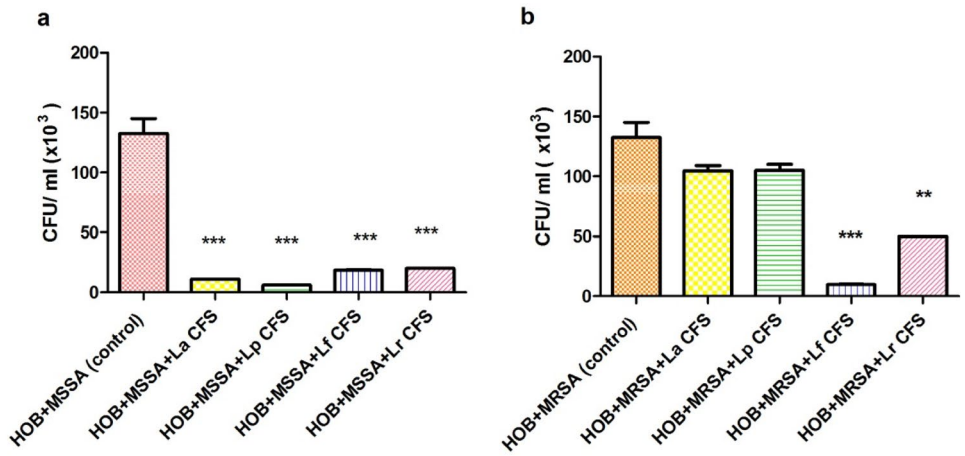

Fig. 3 Effects of different lactobacilli CFSs on invasion of *S. aureus* strains. The alterations were determined by comparing with control (HOB cells infected with *S. aureus* strains). **a** Counts of invasive MSSA; **b** Counts of invasive MRSA. The invasion of bacteria in the presence of HOB cells were examined using one-way ANOVA followed by Tukey's multiple comparisons test. **,***: Significant at $P < 0.01$ level and $P < 0.0001$ level values, respectively

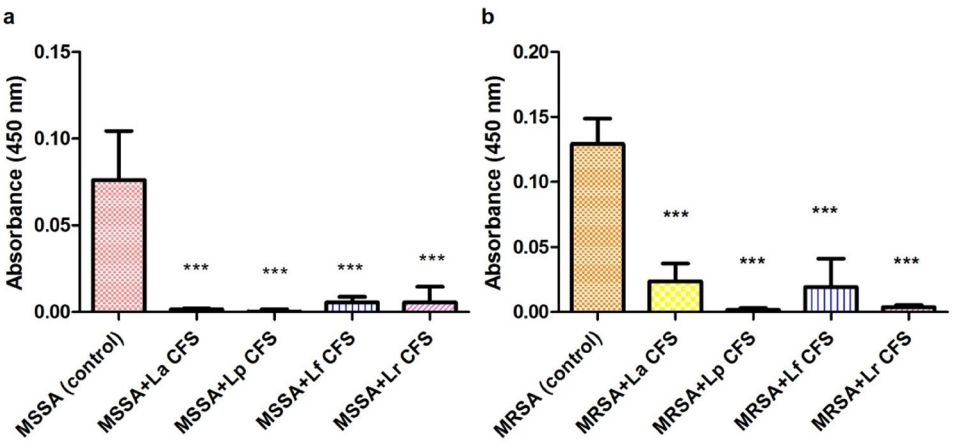

Fig. 4 Effects of different lactobacilli CFSs on biofilm of *S. aureus* strains. The alterations were determined by comparing with control (*S. aureus* strains). **a** Biofilm alterations of MSSA; **b** Biofilm alterations of MRSA. The biofilm formation of bacteria in the presence of CFSs was examined using one-way ANOVA followed by Tukey's multiple comparisons test ***: Significant at $P < 0.0001$ level

HOB Cell Viability

We examined the effects of MRSA, MSSA, and all CFSs on the viability of HOB cells on their own. According to these analyses, the viability of HOB cells were shown to be reduced statistically significant in the presence of *S. aureus* strains and CFSs except Lr CFS ($P < 0.0001$). These viability percentages are as follows: MSSA by 40.8 ± 9%, MRSA by 64.3 ± 8%, La by 47.6 ± 9%, Lp by 34.3 ± 9%, and Lf by 36.6 ± 8%) (Fig. 5).

We also examined the effects of *S. aureus* in the presence of all CFSs on the viability of HOB cells co-existence conditions. According to these analyses the viability of HOB cell during co-incubation with MSSA and CFSs was shown to be decreased significantly. The viability percentages are as follows: La by 83.7 ± 4%, Lp by 96 ± 4%, Lf by 65.3 ± 12%, and Lr by *53.7* ± 14%. However, during co-incubation with MRSA, the pre-treatment of HOB cells with CFSs did not alter viability significantly (Fig. 6a, b).

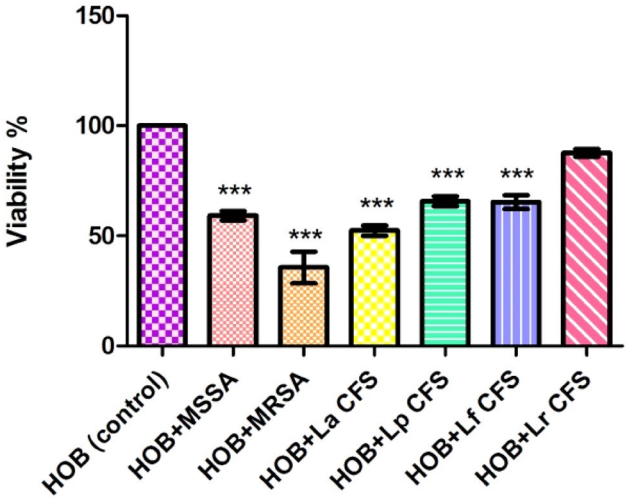

Fig. 5 Effects of different lactobacilli CFSs and *S. aureus* strains on HOB cell viability. The alterations were determined by comparing with control (HOB cells). The cell viability in the presence of CFSs and/or bacteria were examined using one-way ANOVA followed by Tukey's multiple comparisons test ***: Significant at $P < 0.0001$ level

Discussion

In our study, CFSs obtained from different Lactobacillus species were examined for the potential role of bioactive compounds in the supernatants on growth, adhesion, and invasion of *S. aureus* in HOB cell culture and on the cell viability of the HOB cell line. *L. plantarum, L. fermentum, L. acidophilus*, and *L. rhamnosus* which we used in our experiments are the mostly investigated strains for their possible inhibitory effects on growth and virulence factors of bacteria [19, 27–35].

It is well known that lactobacilli are able to increase phagocytic activity of human macrophages, to inhibit growth, adhesion, and invasion of pathogens by either competing with them or by changing environmental conditions by producing some bioactive compounds such as bacteriocins, lactic and other organic acids, hydrogen peroxide, and bio-surfactants. These compounds have effects on cell membrane integrity, membrane structure and enzyme activities, and cause DNA damage [16, 17, 36–42].

In the previous studies, it has been shown that different lactobacilli's CFSs inhibited the growth of pathogens such as *E. coli, S. aureus, S. epidermidis, K. pneumoniae, P. fluorescens, P. aeruginosa, S. mutans, L. monocytogenes, V. cholerae,* and Salmonella [30, 43–52]. In our study, we found that CFSs of *L. fermentum* and *L. plantarum* inhibit the growth of MSSA; the growth of MRSA was shown to be reduced in the presence of only *L. acidophilus*'s CFS.

One of the most important virulence factors of bacteria is the ability of adhesion on host cells or medical devices. The inhibitory effects of various lactobacilli's CFSs on adhesion

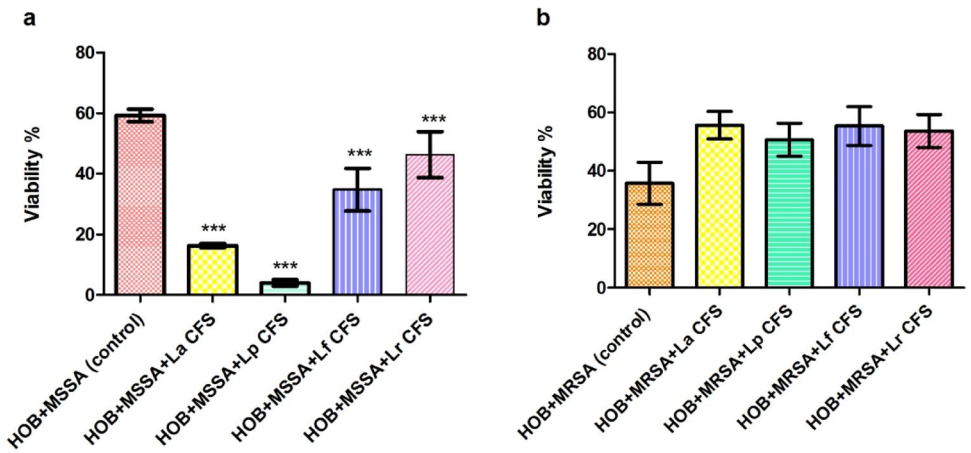

Fig. 6 Alterations of HOB cell viability during co-incubation with different lactobacilli CFSs and *S. aureus* strains. The modulations were determined by comparing with control (*S. aureus*-infected HOB cells). **a** HOB cell viability in the presence of MSSA; **b** HOB cell viability in the presence of MRSA. The cell viabilities were examined using one-way ANOVA followed by Tukey's multiple comparisons test ***: Significant at $P < 0.0001$ level

were investigated in different studies performed in different cell lines [41, 53–55]. Whereas adhesions of *L. monocytogenes*, Salmonella, *S. dysenteriae* and *E. coli* were found to be decreased/inhibited [21, 50, 56], adhesion of *E. coli* O157:H7 and Enteroaggregative *E. coli* were found to be not affected [10, 35, 57]. In our study, we found that the adhesion of MSSA was inhibited in the presence of all lactobacilli's CFSs, but the adhesion of MRSA was enhanced in the presence of CFSs of Lp, Lr, and Lf.

Different lactobacilli's CFSs were shown to prevent different bacterial invasion/internalization process in various cell lines [30, 47, 56, 58–62]. As an example, different CFSs have protective roles against invasion of *L. monocytenes* and Enteroinvasive *E. coli* [20, 50]. Consistent with these studies, we have shown that all the tested lactobacilli's CFSs inhibited the invasion of MSSA, but only CFSs of *L. fermentum* and *L. rhamnosus* were found to inhibit the invasion of MRSA.

Another important virulence factor of *S. aureus* is biofilm formation which is known to increase virulence of microorganism. Many authors reported that biofilm formation of *S. aureus, Candida albicans, P. aeruginosa, E. coli, S. mutans, B. cereus*, and Vibrio [33, 34, 52, 63–66] was inhibited in the presence of CFSs of different Lactobacillus species. Consistent with these studies, we have also shown that all four CFSs have effectively found to inhibit the biofilm formation of both *S. aureus* strains. All these results indicate that lactobacilli CFSs have anti-biofilm effects on pathogens.

When bacteria infect host cells, viabilities of both host cells and bacterium alter. In our study, the individual effects of MSSA, MRSA, and all CFSs on HOB cell viability were examined. In addition, we also investigated the viability of MSSA and MRSA-infected HOB cell line in the presence of CFSs. Various studies have showed that CFSs obtained from different lactobacilli have toxic activity on different cell lines such as HT-29, Caco-2, HeLa, MCF-7, and AGS [67–69]. Consistent with these studies, in our study, all lactobacilli's CFSs except *L. rhamnosus* inhibited the viability of HOB cell line. As some authors suggest that this inhibitory effect is related with decreased pH levels and increased lactic acid levels provided by lactobacilli CFSs [67, 68, 70].

We also found that infection of HOB cells with both two *S. aureus* strains has also significant inhibitory effect on the viability of HOB cells. In consistent with previous studies, the viability of MRSA-infected HOB cells were shown to be not altered significantly in the presence of all tested CFSs [41, 58, 61, 71]. In contrast with these results, MSSA-infected HOB cells viabilities were shown to be inhibited in the presence of CFSs when compared to MRSA-infected HOB cells.

In consistent with previous studies, we found an antagonist relationship between lactobacilli and *S. aureus*. In our study, the growth alterations of *S. aureus* were investigated in HOB cell culture; as far as we know, this is the first study examining the effects of lactobacilli's CFSs on growth of *S. aureus* in cell culture. The most important result is that all CFSs were found to be less effective for inhibiting the antibiotic resistant *S. aureus* strain representing as the problematical bacteria. These results indicate that antibiotic resistance become a major concern during the selection of the pathogenic strains for using in the experiments investigating the inhibitory potentials of probiotics.

There are some limitations in the present study such as the growth alterations of pathogenic bacteria are tested in live cell condition (HOB cell line). As the incubation of MRSA and MSSA were not prolonged for 24 h, growth curve was not able to be detected in order to avoid deleterious effect on the HOB cells.

HOB cell and *S. aureus* interactions are not yet clearly known. The knowledge of invasion ability of *S. aureus* in cell culture assays is limited in the literature and to clarify the difference between adhesion and invasion capacity of MRSA, future investigations with different cell lines are needed. In the present study, we found that MRSA and MSSA strain had different cytotoxic effects on HOB cells. We chose these strains according to their different antibiotic resistance properties, but the other biological characteristics of them (different virulence mechanisms, etc.) may be major determinatives.

Author Contributions Concept: FKY, DG, and MAK. Data collection & Processing: FKY, DG, and GİG. Analysis & Interpretation: FKY, DG, and GİG. Literature Research: FKY, DG, and MAK. Writing: FKY, DG, and MAK. Critical Reviews: FKY, DG, GİG, and MAK.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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