# NEW APPROACHES TO THE TREATMENT OF DRUG-RESISTANT BACTERIA

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Today, the treatment of infectious diseases is getting more difficult every day due to increasing drug resistance against microorganisms. In order to overcome the increasing drug resistance globally, a wide variety of studies are carried out on probiotic microorganisms. In this study, we aimed to investigate the synergistic activities of the bioactive components of *L. casei* and *S. thermophilus* probiotic microorganisms against ESBL-positive *E. coli* and *K. pneumoniae* strains against these two microorganisms. The non-toxic concentrations of *L. casei* and *S. thermophilus* bioactive components in Vero cell culture were determined. Antibacterial activities against ESBL-positive *E. coli* and *K. pneumoniae* at determined non-toxic concentrations were evaluated by studying MIC and MBC concentrations. Firstly, non-cytotoxic concentrations of bioactive metabolites of *S. thermophilus* and *L. casei* were determined on Vero cells. It has been determined that the bioactive metabolites of both *S. thermophilus* and *L. casei* cause toxicity of <32 µg/ml. For this reason, studies have been studied at levels lower than this concentration for antimicrobial efficacy studies. Antimicrobial activities of *L. casei* and *S. thermophilus* alone and in combination against *E. coli* were given. Here, it was determined that the activities of probiotic microorganisms created a synergistic effect when used in combination. It was determined that the activities of *L. casei* and *S. thermophilus*, when used in combination, had a synergistic effect against *K. pneumoniae*. In this study, where we aimed to produce a new and effective drug/solution in the treatment of wounds infected with drug-resistant bacteria (*E. coli* and *K. pneumoniae*), which are very difficult to treat, our findings may be hopeful.

Keywords: drug, resistance, probiotic bacteria

## INTRODUCTION

Today, probiotics are recommended as suitable alternatives to antibiotics for treating diseases (Hossain and Sadekuzzaman, 2017). Probiotics are live microorganisms that can show beneficial effects after ingestion by the host (Araya *et al.*, 2002). Various studies in recent years have shown that oral or topical use of bacterial probiotics can be effective in the treatment of skin disorders (Ye ilova *et al.*, 2012; Argenta *et al.*, 2016; Sekhar *et al.*, 2014; Drago *et al.*, 2011; Nole *et al.*, 2014).

It has been reported in a study that local application of *Streptococcus thermophilus* to the skin can increase the ceramide level of the skin in the epidermis and may be useful in the treatment of atopic dermatitis (Di Marzio *et al.*, 2003). It has also been shown that the use of topical ceramide on its own can accelerate the wound-healing process (Tsuchiya *et al.*, 2013).

Previous studies have reported that some lactic acid bacteria have antagonistic activities against microorganisms that contribute to wound infections, such as Staphylococcus aureus (Besser *et al.*, 2019). Although the efficacy of probiotics in wound treatment has been investigated in various studies, the combined efficacy of the bioactive components of Lactobacilli and streptococci and these bioactive components against ESBL-producing *E. coli* and *K. pneumonia* have not been studied. The most serious problem in *E. coli* and *K. pneumoniae* is drug resistance; these microorganisms secrete broad-spectrum beta-lactamase enzyme. There are severe difficulties in the treatment of infections of these two ESBL-producing microorganisms. Our main aim in

https://doi.org/10.24264/icams-2022.III.10

this study is to develop an effective formulation/drug against ESBL-producing *E. coli* and *K. pneumoniae*.

## MICROORGANISMS

### Probiotic Microorganisms

*Lactobacillus casei, Streptococcus thermophilus*, EBSL positive *Escherichia coli*, ESBL-positive *Klebsiella pneumonia*.

### Probiotic Microorganisms

In the study, *L. casei* and *S. thermophilus* strains were removed from -80 ºC, dissolved in a 37 ºC water bath, then disbanded in Tryptic Soy Broth (TSB) medium and incubated in an incubator with 5% carbon dioxide for 48 hours. At the end of the incubation, 100 µl of bacterial strains were taken and passaged into Mueller-Hinton agar. At the 24$^{th}$ hour of incubation following the passage, bacterial strain verification was performed from the colonies with the help of Gram staining and biochemical tests. Confirmation of the species determination was carried out with biochemical tests and an automated culture system.

### Bioactive Metabolites

Probiotic microorganisms were inoculated from a pure culture sample with the help of a loop into a 500 ml TSB medium. The inoculated TSB culture dishes that were not tightly closed were incubated in a 5% $CO_2$ incubator. At the end of the incubation, the samples taken into 50 ml glass centrifuge tubes were centrifuged at 4000 rpm for 20 minutes and the supernatants were collected. After centrifugation, the collected supernatants were filtered with membrane filters.

### Bacterial Culture: ESBL-producing *E. coli* and *K. pneumoniae* Strains

Cultivation of strains was performed on Eosin Methylene Blue (EMB) medium, antimicrobial tests were performed on Müeller-Hinton broth and Mueller-Hinton agar medium. Cultivations were carried out at 37 °C for 24 hours. Microorganisms were identified with Gram stain, conventional biochemical tests, and automated culture systems when needed.

A healthy cell line (Vero cell line) was used for the detection of non-toxic concentrations of bioactive metabolites of probiotic microorganisms. The vero cell line was obtained from the culture collection of Hatay Mustafa Kemal University Microbiology Cell Culture Laboratory. Stock cultures were taken from the deep freeze and thawed in a water bath pre-warmed to 37 ºC. Then, the cells were incubated in a cell culture medium containing 10% fetal calf serum and antibiotics in a 5% $CO_2$ incubator at 37 °C, and their cultivation was carried out.

### Cell Culture

Evaluations of the cultured cells were carried out daily with an inverted microscope, after the cells covered the surface of the culture dish at a rate of 70-80%, the cells were removed from the surface of the culture dish with trypsinization solution and passaged

https://doi.org/10.24264/icams-2022.III.10

into different culture dishes. For this purpose, the medium in the culture dishes was removed, and the debris was removed by washing the cells with PBS at least twice before the trypsinization solution was added.

After this process, 0.25% trypsinization solution was added to the culture dishes and incubated at 37 ºC for 10 minutes to remove the cells attached to the culture dish surface. The cells were then collected by centrifugation at 1500 rpm for 10 minutes. Then, the cell pellet to be resuspended in RPMI-1640 medium was counted and the cell density was adjusted to $1x10^6$ cells.

**Determination of Bioactive Metabolite Doseages**

Flat-bottomed 96-well microplates were used to determine the non-toxic doses of bioactive metabolites obtained from *L. casei* and *S. thermophilus* bacteria. Different concentrations of bioactive metabolites were studied.

The bioactive components at these concentrations were added to the media of Vero cells and the cells were incubated under the same conditions. Cytotoxic/non-cytotoxic concentrations of bioactive components were determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) analysis method after the cells were incubated for 24 hours.

**MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] Method**

The cytotoxic concentrations of these bioactive metabolites on healthy cells were determined by the MTT method, which is a calorimetric and quantitative method. The MTT test was performed in flat-bottomed 96-well plates by adjusting the cell density to $1x10^6$ cells/ml (Mossman, 1983).

It was incubated for 6 hours for the adhesion of cells to the bottom of the plate (at 37 °C and 5% $CO_2$). Thus, the metabolic activity of the incubated cells was achieved to reach high levels. During this time, serial dilutions of the biologically active components were prepared (from 1025 µl/ml to 1 µl/ml concentration) and added to 4 wells from each dilution on the adherent cells. At least four wells were determined as cell control and medium control groups.

To determine the effect of bioactive metabolites on cells, the cells were incubated for 24 hours at 37 °C in an incubator with 5% carbon dioxide. At the end of the incubation, 10 µl of MTT was added to each well and the plates were incubated for 4 hours under the same growth conditions.

Absorbance measurements were performed at 570 nm with a spectrophotometer. Proliferation was expressed as the ratio of cells in wells treated with bioactive metabolites to control cells and $IC_{50}$ values were calculated using the SPSS program (SPSS. Inc, Chicago).

**Detection of MIC (Minimum Inhibitory Concentration) and MBC (Minimal Bactericidal Concentration)**

MIC (minimum inhibitory concentration) values of probiotic microorganism bioactive components against *E. coli* and *Klebsiella pneumonia* were determined by the microdilution method. The concentration in which the growth turbidity was not detected visually was accepted as the MIC value.

In addition, 10 µl Mueller-Hinton agar was inoculated from the last dilution in which growth turbidity was observed and all subsequent dilutions were incubated at 37

https://doi.org/10.24264/icams-2022.III.10

°C for 24 hours. The dilution concentration that inhibited bacterial growth by 99% was determined as MBK (minimal Bactericidal Concentration). All experiments were repeated three times.

## RESULTS

Firstly, non-cytotoxic concentrations of bioactive metabolites of *S. thermophilus* and *L. casei* on Vero cells were determined. It was determined that the bioactive metabolites of both *S. thermophilus* and *L. casei* caused toxicity at the level of 16 µg/ml. For this reason, studies were carried out at levels lower than this concentration (16 µg/ml) for antimicrobial efficacy studies (Figures 1 and 2).

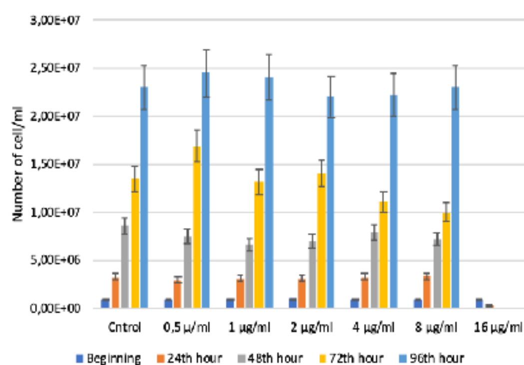


Figure 1. Non-toxic concentrations of bioactive metabolites of *S. thermophilus* on Vero cells

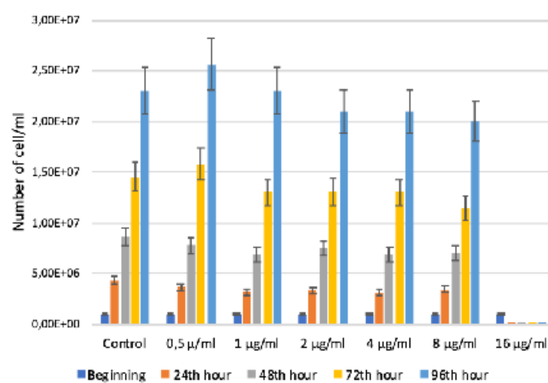


Figure 2. Non-toxic concentrations of bioactive metabolites of *L. casei* in Vero cell line

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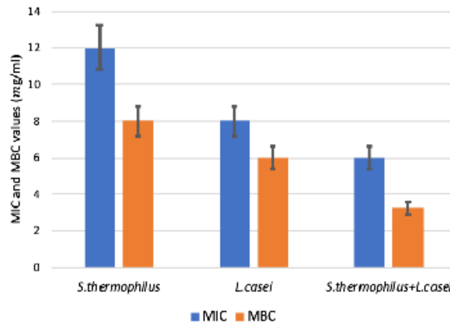


Figure 3. Synergistic activity of *L. casei* and *S. thermophilus* against *E. coli*

Figure 3 shows the antimicrobial activities of single and combined uses of *L. casei* and *S. thermophilus* against *E. coli*. Here, it was determined that the activities of probiotic microorganisms create a synergistic effect when used in combination (Figure 3).

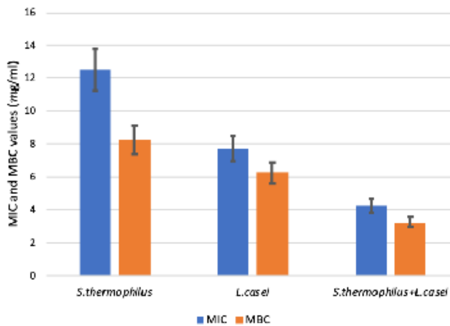


Figure 4. Synergistic activity of *L. casei* and *S. thermophilus* against *K. pneumoniae*

As in Figure 3, MIC and MBC values of single and combined uses of *L. casei* and *S. thermophilus* against *K. pneumonia* are given in Figure 4. As seen from the figure, it has been determined that the activities of both *L. casei* and *S. thermophilus*, when used together, have a synergistic effect against *K. pneumoniae* (Figure 4).

## DISCUSSION

Broad-spectrum beta-lactamases (ESBL) were first identified in the 1980s and detected in *Klebsiella* species and later in *E. coli* and other genera of the *Enterobacteriaceae* family. Antibiotic resistance is a global problem that affects the whole world due to the widespread and incorrect use of antibiotics in the world.

As a result of beta-lactamase, enzyme production is one of the most important resistance mechanisms among *Enterobacteriaceae* members such as *E. coli* and *K. pneumoniae*, which inactivate beta-lactam group drugs by breaking down. To date, hundreds of beta-lactamase enzymes have been identified. It has been reported that nearly half of these enzymes are extended-spectrum beta-lactamases (ESBL, extended-spectrum beta-lactamases).

https://doi.org/10.24264/icams-2022.III.10

ESBLs can be easily transferred between plasmids and bacteria from one bacteria to another. ESBL production in bacteria leads to resistance to cephalosporins and aztreonam. ESBL production, especially seen in Enterobacteriaceae members such as *Klebsiella pneumonia* and *Escherichia coli*, and the easy spread of these enzyme genes to other species have become a serious health problem both in our country and in the world.

Infectious agents created by ESBL-positive strains require a long stay in intensive care units, resulting in serious morbidity and mortality. These microorganisms are among the most important hospital infection agents worldwide and may cause problems in treatment due to multiple antibiotic resistance (Kaftandzieva *et al*., 2014).

## CONCLUSION

In the present study, we showed that probiotic microorganism metabolites can be beneficial in overcoming bacterial resistance against ESBL strains, which is one of the most crucial resistance problems in bacteria. We think that when these results are supported by advanced studies, they can be an important alternative solution for a serious problem for the whole world.

## REFERENCES


Araya, M., Morelli, L., Reid, G., Sanders, M., Stanton, C. and Pineiro, M. (2002), *Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food*, London, Canada: World Health Organization, Food, and Agriculture Organization of the United Nations.

Argenta, A., Satish, L., Gallo, P., Liu, F. and Kathju, S. (2016), “Local Application of Probiotic Bacteria Prophylaxes against Sepsis and Death Resulting from Burn Wound Infection”, *PloS one*, 11(10), e0165294, https://doi.org/10.1371/journal.pone.0165294.

Besser, M., Terberger, J., Weber, L., Ghebremedhin, B., Naumova, E.A. and Arnold, W.H. (2019), “Impact of Probiotics on Pathogen Survival in an Innovative Human Plasma Biofilm Model (*hp*BIOM)”, *Journal of Translational Medicine*, 17(1), 243, https://doi.org/10.1186/s12967-019-1990-4.

Di Marzio, L., Cinque, B., Cupelli, F., De Simone, C., Cifone, M.G. and Giuliani M. (2008), “Increase of skin-ceramide levels in aged subjects following a short-term topical application of bacterial sphingomyelinase from Streptococcus thermophilus”, *International Journal of Immunopathology and Pharmacology*, 21, 137-143, https://doi.org/10.1177/039463200802100115.

Drago, L., Iemoli, E., Rodighiero, V., Nicola, L., De Vecchi, E. and Piconi, S. (2011), “Effects of *Lactobacillus salivarius* LS01 (DSM 22775) Treatment on Adult Atopic Dermatitis: A Randomized Placebo-Controlled Study”, *International Journal of Immunopathology and Pharmacology*, 24(4), 1037-1048, https://doi.org/10.1177/039463201102400421.

Hossain, M.I., Sadekuzzaman, M. and Ha, S.D. (2017), “Probiotics as Potential Alternative Biocontrol Agents in the Agriculture and Food Industries: A Review”, *Food Research International*, 100(1), 63-73, https://doi.org/10.1016/j.foodres.2017.07.077.

Kaftandzieva, A., Trajkovska-Dokic, E., Kotevska, V., Cekovska, Z. and Jankoska, G. (2014), “Genotypes of ESBL Producing *Escherichia coli* and *Klebsiella pneumoniae* about Resistance to Antimicrobial Drugs”, *Prilozi*, 35(2), 31-38, https://doi.org/10.2478/prilozi-2014-0004.

Mosmann, T. (1983), “Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays”, *Journal of Immunological Methods*, 65, 55-63, https://doi.org/10.1016/0022-1759(83)90303-4.

Nole, K.L.B., Yim, E. and Keri, J.E. (2014), “Probiotics and Prebiotics in Dermatology”, *Journal of the American Academy of Dermatology*, 71(4), 814-821, https://doi.org/10.1016/j.jaad.2014.04.050.

Sekhar, M.S., Unnikrishnan, M., Vijayanarayana, K., Rodrigues, G.S. and Mukhopadhyay, C. (2014), “Topical Application/Formulation of Probiotics: Will It Be a Novel Treatment Approach for Diabetic Foot Ulcer?”, *Medical Hypotheses*, 82(1), 86-88, https://doi.org/10.1016/j.mehy.2013.11.013.

Tsuchiya, S., Ichioka, S., Sekiya, N., Tajima, S., Iwasaki, T. and Numata, S. (2013), “The Effect of a Hydrocolloid Dressing Containing Ceramide-2 on Split-Thickness Wounds in a Laser-Induced Erosion Model”, *Advances in Skin and Wound Care*, 26(5), 224-229, https://doi.org/10.1097/01.ASW.0000428952.00149.77.

Ye ilova, Y., Çalka, Ö., Akdeniz, N. and Berkta , M. (2012), “Effect of Probiotics on the Treatment of Children with Atopic Dermatitis”, *Annals of Dermatology*, 24(2), 189-193, https://doi.org/10.5021/ad.2012.24.2.189.


https://doi.org/10.24264/icams-2022.III.10