Effect of the combination of *Lactobacillus acidophilus* (probiotic) with vitamin K3 and vitamin E on *Escherichia coli* and *Staphylococcus aureus*: An *in vitro* pathogen model

OZGUR CELEBI[1], ALI TAGHIZADEHGHALEHJOUGHI[2], DEMET CELEBI[3,4], ROBIN MESNAGE[5], KIRILL SERGEEVICH GOLOKHVAST[6], ANDREEA LETITIA ARSENE[7], DEMETRIOS A. SPANDIDOS[8] and ARISTIDIS TSATSAKIS[9]

[1]Department of Medical Microbiology, Faculty of Medicine, Ataturk University, 25240 Erzurum; [2]Department of Medical Pharmacology, Faculty of Medicine, Seyh Edebali University, 11000 Bilecik; [3]Department of Microbiology, Faculty of Veterinary Medicine and [4]Vaccine Application and Development Center, Ataturk University, 25240 Erzurum, Turkey; [5]Department of Medical and Molecular Genetics, Faculty of Life Sciences and Medicine, King's College London, London WC2R 2LS, United Kingdom; [6]Russian Academy of Education, Moscow, 119121, Russia; [7]Department of General and Pharmaceutical Microbiology, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, 020021 Bucharest, Romania; [8]Laboratory of Clinical Virology and [9]Department of Forensic Sciences and Toxicology, Faculty of Medicine, University of Crete, 71003 Heraklion, Greece

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Abstract. The gut microbiota plays a key role in maintaining health and regulating the host's immune response. The use of probiotics and concomitant vitamins can increase mucus secretion by improving the intestinal microbial population and prevent the breakdown of tight junction proteins by reducing lipopolysaccharide concentration. Changes in the intestinal microbiome mass affect multiple metabolic and physiological functions. Studies on how this microbiome mass and the regulation in the gastrointestinal tract are affected by probiotic supplements and vitamin combinations have attracted attention. The current study evaluated vitamins K and E and probiotic combinations effects on *Escherichia coli* and *Staphylococcus aureus*. Minimal inhibition concentrations of vitamins and probiotics were determined. In addition, inhibition zone diameters, antioxidant activities and immunohistochemical evaluation of the cell for DNA damage were performed to evaluate the effects of vitamins and probiotics. At the specified dose intervals, *L. acidophilus* and vitamin combinations inhibit the growth of *Escherichia coli* and *Staphylococcus aureus*. It could thus contribute positively to biological functions by exerting immune system-strengthening activities.

Correspondence to: Dr Demet Celebi, Department of Microbiology, Faculty of Veterinary Medicine, Ataturk University, University Street, 25240 Erzurum, Turkey
E-mail: dmtdl2006@gmail.com

Dr Ali Taghizadehghalehjoughi, Department of Medical Pharmacology, Faculty of Medicine, Seyh Edebali University, 27 Fatih Sultan Mehmet Street, 11000 Bilecik, Turkey
E-mail: ali.tgzd@bilecik.edu.tr

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Introduction

The intestinal microbiota constitutes the most important part of the human microbiota. It is considered that ~1,000 different bacteria are in each healthy individual's colon mucosa and feces. The microbiota serves a role in a variety of diseases, including metabolic disorders, inflammatory and autoimmune diseases, allergies and even conditions where microbiome involvement seems implausible (1-5).

Escherichia coli and *Staphylococcus aureus*, intestine flora, cause adverse effects on intestinal permeability by increasing their numbers in the intestinal microbiota in dysbiosis. The alpha toxin of *Staphylococcus aureus* alters intestinal integrity and impairs the barrier function of intestinal cells *in vitro* (6). *Staphylococcus aureus* serves a role in inflammatory bowel disease, as evidenced by the fact that gut-derived *Staphylococcus aureus* superantigens can induce inflammatory responses. A number of studies have shown that the colon can be a reservoir of antibiotic resistance genes. For example, vancomycin-resistant *Staphylococcus aureus* colonizes the intestinal tract. In previous studies, it has been observed that the intestinal transport of *Staphylococcus aureus* is increased among hospitalized patients and infants (7-13). *Escherichia coli* is gram-negative and some *E. coli* strains provide vitamin K and vitamin B12, which are beneficial for the host (14). However, colitis and other intestinal diseases develop as a result of the increase in the Enterobacteriaceae

family and especially *Escherichia coli* as a result of high-fat contents, diet, inactivity, and unnecessary and incorrect antibiotic use (15). Especially in patients with immunosuppressed colon cancer, changes in the flora cause bad results in a whole spectrum from metabolic diseases to neurological diseases.

An increase in the pathogenic bacteria population, especially *E. coli* and *S. aureus* in the intestine tract leads to an increase in the incidence of tumor appearance. Mitochondria contain DNA called mtDNA. As with nuclear DNA, the presence of endogenous reactive oxygen species (ROS) causes mtDNA damage. ROS is one of the well-known parameters in cancer formation. A number of studies show that chronic ROS formation induces RNA and DNA break and leads to cancer formation. 8-OHdG and H2AX are markers to evaluate the RNA and DNA breakout (16-18).

The capacity of Lactobacilli to adhere to epithelial cells of the host intestinal tract is crucial in inhibiting enteropathogenic infections. Species of *Lactobacillus*, such as *L. acidophilus, L. helveticus, L. rhamnosus, L. case, L. paracasei, L. reuteri*, and *L. fermentum*, compete with enteric pathogens for binding sites, such as Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), and, a new pathotype, adherent-invasive *E. coli* (AIEC) (19-24). In addition to probiotics, vitamin E, which is stated to have a significant effect on the colon, acts as an important antioxidant (against lipid peroxidation) (25). The use of probiotics can improve the intestinal microbial population, increase mucus secretion, and reduce the number of lipopolysaccharides (LPSs) to prevent the breakdown of tight junction proteins. When LPS binds endothelial cells to toll-like receptors (TLR) 2 and 4, dendritic cells and macrophage cells are activated and inflammatory markers increase. Furthermore, a reduction in intestinal dysbiosis and intestinal leakage after probiotic treatment can minimize the development of inflammatory biomarkers and blunt unnecessary activation of the immune system. By contrast, probiotics enhance the differentiation of T cells towards Th2 and the development of Th2 cytokines such as IL-4 and IL-10 (24-26). Vitamin K, especially synthetic vitamin K3 (menadione), is twice as strong as natural vitamins K1 and K2. K3 is produced by bacteria in the intestinal microflora and serves an important role in blood coagulation (26). HT-29 and Caco-2 cells derived from human colorectal carcinoma are the most commonly used in numerous *in vitro* cell culture absorption models.

To date, few studies have investigated the ability of *lactobacilli* to inhibit the adhesion of *Escherichia coli* and *Staphylococcus aureus* to intestinal epithelial cells using methods evaluating the incorporation of vital dyes or the inhibition of cell colony formation (27-30). The present study determined the effect of the *Lactobacillus acidophilus* ATCC 1356 strain and vitamins K3 and E on *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* (MRSA) infection in the human colon adenocarcinoma cell line grade one HT-29 and Caco-2.

Materials and methods

Chemicals and reagents. Vitamin K3, vitamin E, 9% isotonic sodium chloride solution, tryptic soy broth, ethanol, blood agar, agar, crystal violet solution, Dulbecco modified Eagles medium (DMEM), fetal calf serum (FBS), phosphate buffer solution (PBS), antibiotic antimitotic solution (100X), L glutamine, and trypsin-EDTA were obtained from MilliporeSigma.

Bacterial strains. Lactobacillus acidophilus ATCC 4356 strains (American Type Culture Collection) were purchased commercially and isolated in the Department of Medical Microbiology, Faculty of Medicine, Ataturk University. The *Escherichia coli* ATCC 25922, MRSA ATCC 29213 strain, and *Lactobacillus acidophilus* ATCC 4356 strains were used in the present study. *Lactobacillus acidophilus* ATCC 4356 was incubated in Mann, Rogosa, and Sharpe broth (MRS Broth; MilliporeSigma) at 37˚C and 5% CO_2 for 48 h. *L. acidophilus* was grown in *Lactobacillus* MRS Broth (Himedia) for 24 h at 37˚C in a bacteriological incubator under microaerophilic conditions. The number of cells in suspension was determined with a spectrophotometer (B582; Micronal) at a concentration of 10^7 cells/ml. The optical density and wavelength used were 0.296 and 600 nm for *L. acidophilus*. Cell densities of the inoculum were confirmed by CFU/ml counting after plating in Rogosa agar for *L. acidophilus*. For the preparation of the *L. acidophilus* culture filtrate, 1 ml of the standard suspension was transferred to a Falcon tube containing 6 ml MRS broth and incubated for 24 h at 37˚C in a bacteriological incubator under microaerophilic conditions. Then the broth was centrifuged (2,000 x g for 10 min) at room temperature and filtered through a membrane with a 0.22-μm pore size (Advantec MFS, Inc.). *Escherichia coli* ATCC 25922 strain was used. Bacteria were inoculated into an EMB medium and incubated for 24 h at 37˚C. Then, 10^8 CFU/ml suspension was prepared from the growing colonies according to the McFarland 0.5 chart. The MRSA ATCC 29213 strain was incubated in modified Giolitti and Cantoni broth for 14 h at 37˚C and Baird-Parker plates (supplemented with Giolitti and Cantoni broth 1.5%) for 48 h at 37˚C.

Kirby-Bauer disk diffusion method. Microorganism colonies taken from 24 h cultures were adjusted in sterile saline to McFarland 0.5 turbidity and inoculated into Mueller Hinton medium (meat infusion 2 g/l; casein hydrolysate 17.5 g/l; starch 1.5 g/l; agar-agar 13,0 g/l) with a swab stick. Then, 6 mm diameter Sterile Paper Discs (Oxoid Antibacterial Susceptibility Blank Test Disc; Oxoid Ltd.) were impregnated with 20 μg/ml and placed in Muller Hinton Medium. Zone diameters were measured after 24 h of incubation. Vancomycin was used as the control antibiotic.

Minimal inhibitory concentration. Vitamins E and K were dissolved with Tween 20 to a final concentration of 5 mg/ml. *Escherichia coli* ATCC 25922, MRSA ATCC 29213 and *Lactobacillus acidophilus* ATCC 4356 used in the minimum inhibitory concentration determination method were passed into eosin methylene blue and blood agar and a 24 h fresh culture was prepared by CLSI criteria (31). From the colonies taken from the prepared cultures, a bacterial suspension was prepared in sterile 0.9% saline with the turbidity of *Lactobacillus acidophilus* ATCC 4356, 0.5 McFarland 10^9 CFU/ml, and *Escherichia coli* ATCC 25922, MRSA ATCC 29213, 0.5 McFarland $5x10^5$. Then, 100 μl of tryptic

soy broth medium was added to all wells 1 to 12 of the sterile 96-well microplate. At first, 100 μl of bacterial suspension was added to the 1st well and 100 μl of a 1:1 dilution was added to the 10th well. Secondly, 100 μl of vitamin K, E, and K + E (5-0.007 mg/ml) were added to the 1st well, and 100 μl dilution was made up to the 10th well in a 1:1 ratio (32). For bacteria control only bacterial suspension was added to the well, and only vitamins K and E were added to the 12th well. Then, the microplate was covered with parafilm and incubated for 24 h at 37˚C. Vancomycin was used as the control antibiotic (33).

Cell culture infection model

Colon cancer cell lines. The human colon cancer cell lines HT-29 (ATCC no: HTB-38) and Caco-2 (ATCC no: HTB-37) were used. Frozen cells were rapidly thawed and centrifuged at 200 x g at room temperature for 5 min. Cells were collected in a 25 cm^2 flask by adding fresh DMEM high glucose, 10% FBS and 1% antibiotics (penicillin, streptomycin, and amphotericin B). When 80% of the flasks were covered with cells, they were centrifuged at 200 x g at room temperature for 5 min (by removing trypsin-ethylenediaminetetraacetic acid (EDTA; 0.25% trypsin-0.02% EDTA)). The supernatant was discarded, and the cell suspension was seeded at 10^4 cells/well in 96-well cell culture plates.

Preparation of cell culture samples. Vitamins E and K, an MRSA strain, and a *Lactobacillus acidophilus* strain were used in the present study. Groups were: Control group, the group containing DMSO (dimethyl sulfoxide) was used as a positive control group; vitamin E (5 mg/ml), vitamin K3 (5 mg/ml), *Lactobacillus acidophilus* (10^9 CFU/ml), *Escherichia coli* ATCC 25922, (10^8 CFU/ml) and MRSA ($5x10^5$ CFU/ml). The experiment began when the cells in the plates reached a density of 85-90%. A total of 10 replicates were used for each dose (n=10).

MTT analysis (cytotoxicity analysis). When the experiment had finished, 10 μl of MTT solution was added to each well and incubated for 4 h at 37˚C with 5% CO_2. To dissolve the formed formazan crystals, 100 μl of dimethyl sulfoxide (DMSO) solution was added to the wells. Cell viability (%) was read using a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Inc.) at 570 nm. The viability rates were compared with those of the control group.

Oxidative stress markers. To measure the oxidative stress level, the total antioxidant capacity (TAC) and the total oxidant status (TOS) were determined with a commercial kit (Rel Assay Diagnostics) according to the manufacturer's instructions. TAC levels were examined at 660 nm and TOS at 530 nm in a Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Inc.).

Immunohistochemistry. Cultured cells were incubated for 30 min in paraformaldehyde (4% and room temperature) solution. The cells were then incubated in 3% H_2O_2 for 5 min. A 0.1% Triton-X solution was dripped onto the cells, washed with PBS, and left for 15 min. After incubation, serum-free blocking buffer (cat. no. X090930-2; Agilent Technologies, Inc.) was dripped onto the cells and kept in the dark for 5 min at room temperature. Then, the primary antibody (8-OHdG cat. no. sc-66036; Santa Cruz Biotechnology, Inc.; 1:100) was added dropwise and incubated in accordance with the manufacturer's protocols. An immunofluorescence secondary antibody was used as a secondary marker (FITC; cat. no. ab6785; Abcam) and incubated in the dark for 45 min at room temperature. The cells were stained with Texas Red (cat. no. ab6787; Abcam; 1:1,000) in the dark for 45 min. Then, DAPI with mounting medium (cat. no. D1306; Thermo Fisher Scientific, Inc.; 1:200) was dropped onto the sections and they were kept in the dark for 5 min. Then, the sections were closed with a coverslip. The stained sections were examined under a fluorescence microscope (Zeiss Axio; Zeiss AG).

Statistical analyses

Cell culture. The results are given as the mean ± standard error of the mean, a visual representation of statistical quantities estimated from the data with assumptions about the underlying distribution of the data obtained in box plot charts. The statistical comparison of the groups with each other was calculated by one-way ANOVA and Tukey's HSD method. One-way ANOVA calculations to be used in statistical analysis were performed with SPSS 20 software (IBM Corp.).

Immunohistochemistry. To determine the intensity of positive staining from the images captured from the stained samples, five random areas were selected from each image and evaluated using the Zen Imaging Software program (Zeiss AG). Data was statistically defined as the mean ± standard deviation for the percentage area. The Mann-Whitney U test was performed to compare positive immunoreactive cells and immunopositive stained areas with healthy controls. The data are presented as the mean ± standard deviation.

P<0.05 was considered to indicate a statistically significant difference.

Results

Vitamins and probiotics are frequently consumed for their alleged health benefits. However, it is not known whether they can interact and influence the health effects of each other. The present study documented the interactions between vitamins E and K with *Lactobacillus acidophilus* against MRSA or *Escherichia coli*.

Effects on bacterial growth. The present study first assessed if the different combinations of vitamins exerted toxic effects on bacterial growth. After the concentrations of bacteria and probiotics were prepared, the doses of vitamins were adjusted and inoculations were made. The minimal inhibitory concentration (MIC) value of vitamin E on MRSA was 1.25 mg/ml, and *Lactobacillus acidophilus* + vitamin E did not show any MIC value. By comparison, the MIC value of MRSA + *Lactobacillus acidophilus* + vitamin E was 1.25 mg/ml. While no effect was observed in vitamin K MRSA, MIC values of *Lactobacillus acidophilus* + vitamin K were found to be 1.25 mg/ml, and MRSA + *Lactobacillus acidophilus* + vitamin K MIC values were determined to be 2.5 mg/ml. The MIC values of *Lactobacillus acidophilus* + vitamins E + K against MRSA or *Escherichia coli* are

Table I. Minimal inhibitory concentration values of vitamins in bacteria.

	Strain mg/ml				
Vitamin	*E. coli*	MRSA	*Lactobacillus acidophilus*	*E. coli* + *Lactobacillus acidophilus*	MRSA + *Lactobacillus acidophilus*
Vitamin E	0.03	1.25	>5	0.03	1.25
Vitamin K	>5	>5	1.25	2.5	2.5
Vitamin E + K	0.02	0.007	0.009	2.5	0.15

MRSA, methicillin-resistant *Staphylococcus aureus*.

Table II. *Escherichia coli* and MRSA zone diameters at 6, 12, 18, and 24 h (mm).

	Time			
Agent	6 h	12 h	18 h	24 h
Vitamin E	No zone[a,b]	2[a], 5[b]	6[a], 12[b]	11[a], 17[b]
Vitamin K	No zone[a,b]	No zone[a,b]	No zone[a,b]	No zone[a,b]
Vitamin E + vitamin K + *Lactobacillus acidophilus*	No zone[a,b]	7[a],11[**]	15[a], 17[**]	17[a], 23[b]

[a]*Escherichia coli* and [b]MRSA. The strongest inhibition zone occurred at 24 h. MRSA, methicillin-resistant *Staphylococcus aureus*.

shown in Table I. Altogether, the combination of vitamin E and K had the most potent effects and strongly inhibited bacterial growth when *Lactobacillus acidophilus* against MRSA or *Escherichia coli* was grown in isolation. The MIC values were larger when the bacteria were co-cultured.

Kirby Bauer disk diffusion results. In the present study, an antibiogram was performed by inoculating *Escherichia coli* and MRSA suspension into Mueller Hinton medium. The inhibition zones of discs impregnated with vitamins and probiotics were measured at 6, 12, 18, and 24 h. The most effective after 24 h was the vitamin E + vitamin K + *Lactobacillus acidophilus* combination against *Escherichia coli* by opening a 23 mm zone against 17 mm MRSA. The inhibition results against *Escherichia coli* and MRSA are shown in Table II. The results show a time-dependent change in zone diameter, which was the most pronounced with vitamin E.

Cell culture results

MTT assay. The present study then examined how probiotic and vitamin supplementation in HT-29 and Caco-2 colon cancer cells exposed to the bacteria would affect the viability of the cells. The MTT results obtained in the *Escherichia coli* and MRSA on Caco and HT-29 cell lines are presented in Fig. 1. No significant difference was found between the *Lactobacillus acidophilus* control group and the control group. When Escherichia coli control and control groups were compared, the viability rate was found to be 86.36%. When *Lactobacillus acidophilus* + vitamin K 1.25 mg/ml, vitamin E + *Escherichia coli* 0.03, and vitamin E + vitamin K + *Escherichia coli* 0.02 groups were compared with the control group, the lowest viability rates were found with 64.4% ($^{**}P<0.001$), and 69.2% ($^{*}P<0.05$), respectively. Regarding MRSA, no significant difference was found between the control groups and the positive control group. When the MRSA control and control groups were compared, the viability rate was found to be 83.7%. When the *Lactobacillus acidophilus* + vitamin K 1.25 mg/ml + vitamin E 0.02 groups were compared with the control group, the lowest viability rates were found at 67.4%, respectively, and these values were statistically significant. ($^{**}P<0.001$).

L. acidophilus did not decrease Caco cell viability similar to the *E. coli* control group (P>0.05). By contrast, the bacteria and vitamin combination, especially in the vitamin E group, decreased the viability (P>0.05) but the results were not statistically significant. E. *coli* + *L. acidophilus* + vitamin E decreased cancer cell viability by nearly 20% compared with the control group. The highest toxicity was noticed in *E. coli* + *L. acidophilus* + vitamin E + vitamin K near 25% (P<0.001). The MTT results are shown in Fig. 1. *L. acidophilus* did not decrease Caco cell viability, a result similar to MRSA treatment. Bacteria and vitamin combination, especially in the vitamin E group, decreased viability (P<0.05). MRSA + *L. acidophilus* + vitamin E decreased cancer cell viability by nearly 25% compared with the control group (P<0.05). The highest toxicity was seen in MRSA + *L. acidophilus* + vitamin E + vitamin K, by nearly 29% (P<0.001).

TAC and TOS measurement. The present study then evaluated whether antioxidant capability or oxidative stress was changed by exposure to probiotics, vitamins, or their combination. There was no significant difference between TAC and TOS levels in the DMSO group in HT-29 cells treated with *E. coli* compared with the control group. TAC levels of the control group and *Lactobacillus acidophilus* control group,

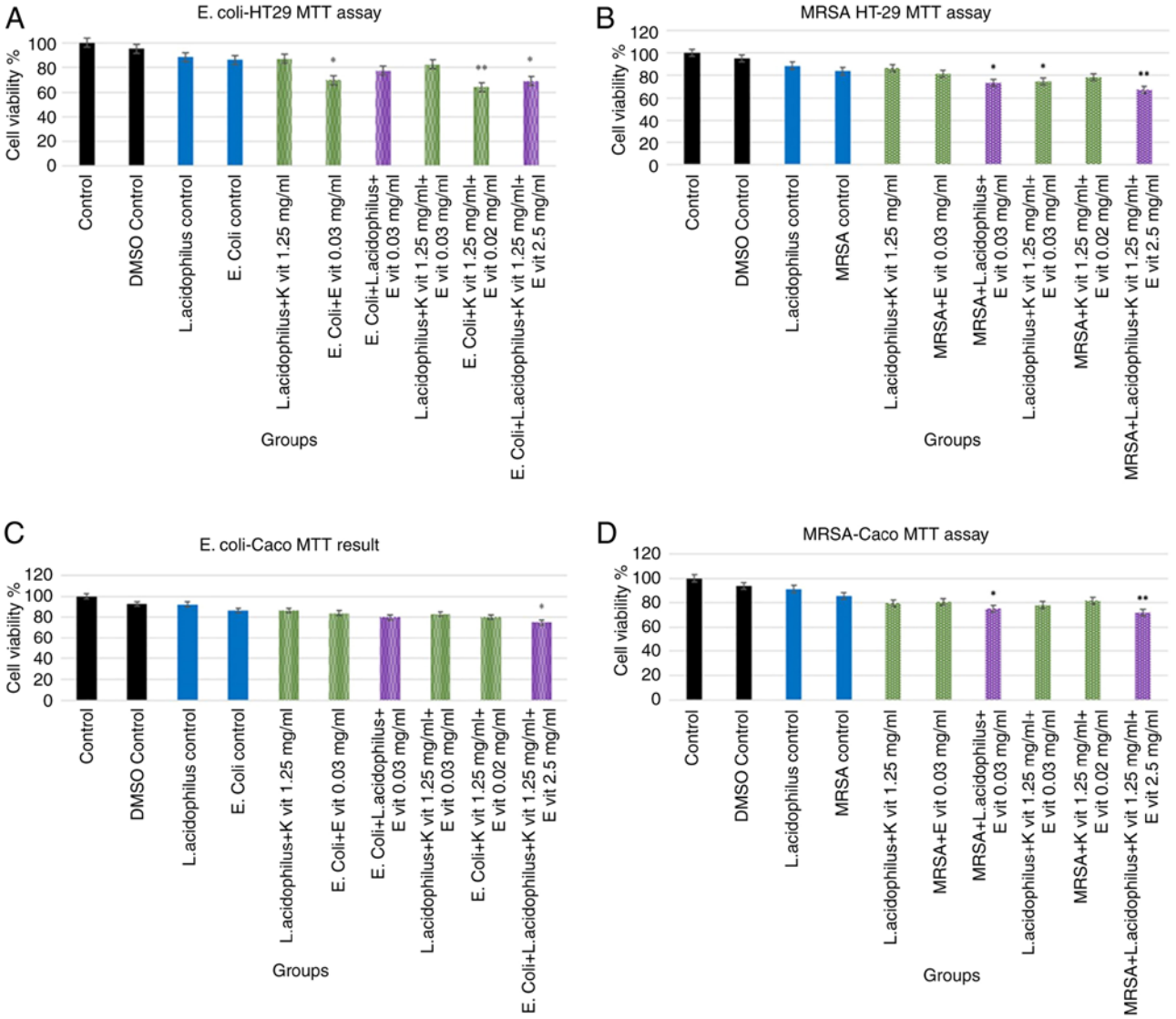

Figure 1. MTT (Cytotoxicity) assay test. MTT assay results for (A) *Escherichia coli*-HT29, (B) MRSA-HT29, (C) *E. coli*-Caco-2, and (D) MRSA-Caco-2 in cell culture after 24 h. *P<0.05 and **P<0.001 vs. with the control group. MRSA, methicillin-resistant *Staphylococcus aureus*; K vit, vitamin K; E vit, vitamin E.

Lactobacillus acidophilus + vitamin K 1.25, *Escherichia coli* + vitamin E 0.03, and *Escherichia coli* + vitamin K + vitamin E 0.02 were significantly higher (**P<0.001). TOS values of these groups were significantly lower compared with the control group (**P<0.001, *P<0.05).

There was no significant difference between TAC and TOS levels in the DMSO group in HT-29 cells given MRSA compared with the control group. The TAC levels of the control group and the L. *acidophilus control*, *L. acidophilus* + vitamin K 1.25, and *L. acidophilus* + vitamin K + vitamin E + 1.25 groups were significantly higher (**P<0.001). The TOS values of these groups were significantly lower than those of the control group (**P<0.001, *P<0.05). In addition, *E. coli* + *L. acidophilus* + vitamin E affected antioxidant capacity compared with the control group (P<0.05). The antioxidant capacity decreased by adding vitamin E to both L. acidophilus (P<0.05) and MRSA compared with the bacteria control group. while E and vitamin K combination with bacteria lead to a decreased antioxidant significantly in *E. coli* + vitamin E + K (P<0.05) and *E. coli* + *L. acidophilus* + vitamin E + vitamin K group 2 Trolox Equiv mmol/l^{-1} (P<0.001). *E. coli* + vitamin E + K (P<0.05) and *E. coli* + *L. acidophilus* + vitamin E + vitamin K group increased the oxidative status significantly (P<0.05) compared with the control group. The oxidative status results are shown in Fig. 2. The DMSO control did not affect the antioxidant and oxidant status. In addition, the antioxidant capacity decreased by adding vitamin E to both *L. acidophilus* (P<0.05) and MRSA in comparison to the control group. The combination of vitamin E and K with bacteria led to decreased antioxidant capacity and increased oxidant status in the Caco-2 culture. The antioxidant capacity primarily decreased in MRSA + *L. acidophilus* + vitamin E + vitamin K group 2 Trolox equiv mmol/l^{-1} (P<0.001). Oxidant status increased slightly by adding vitamins to bacterial species (P>0.05).

Immunohistochemistry results. Whether the change in oxidative status could be linked to the development of DNA damage was evaluated. For the control group, in immunofluorescent staining, negative 8-OHdG, and H2A.X expressions were established. For the *E. coli* control group, the immunofluorescent

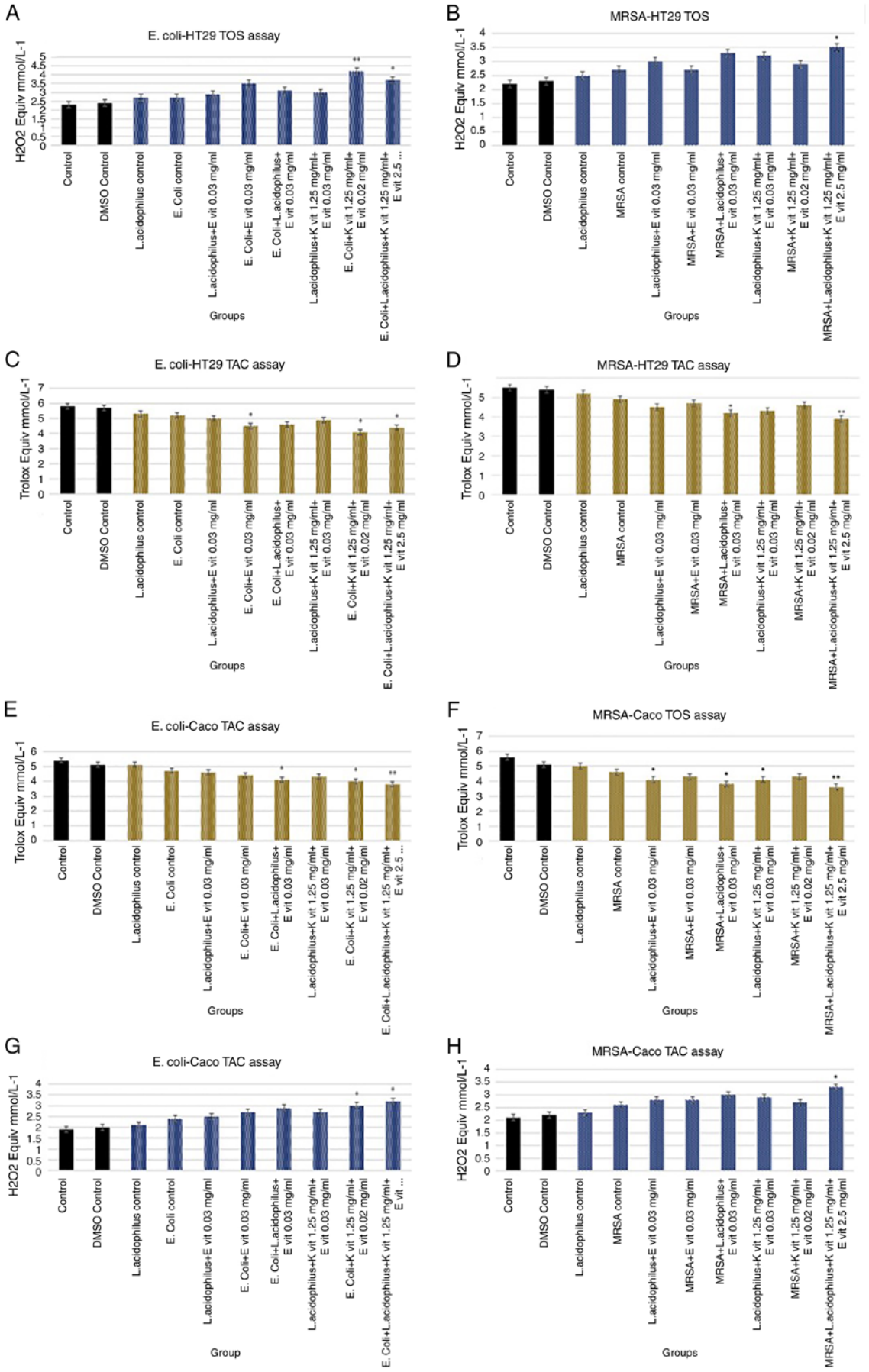

Figure 2. Antioxidant and oxidant activity measurement. TAC and TOS assay results of (A) *Escherichia coli*-HT29 TOS, (B) MRSA-HT29 TOS, (C) *E. coli*-HT29 TAC, (D) MRSA-HT29 TAC, (E) *E. coli*-Caco-2 TAC, (F) MRSA-Caco-2 TOS, (G) *E. coli*-Caco-2 TOS and (H) MRSA-Caco-2 TAC in cell culture after 24 h. *P<0.05 and **P<0.001 vs. with the control group. TAC, total antioxidant capacity; TOS, total oxidant status; MRSA, methicillin-resistant *Staphylococcus aureus*; K vit, vitamin K; E vit, vitamin E.

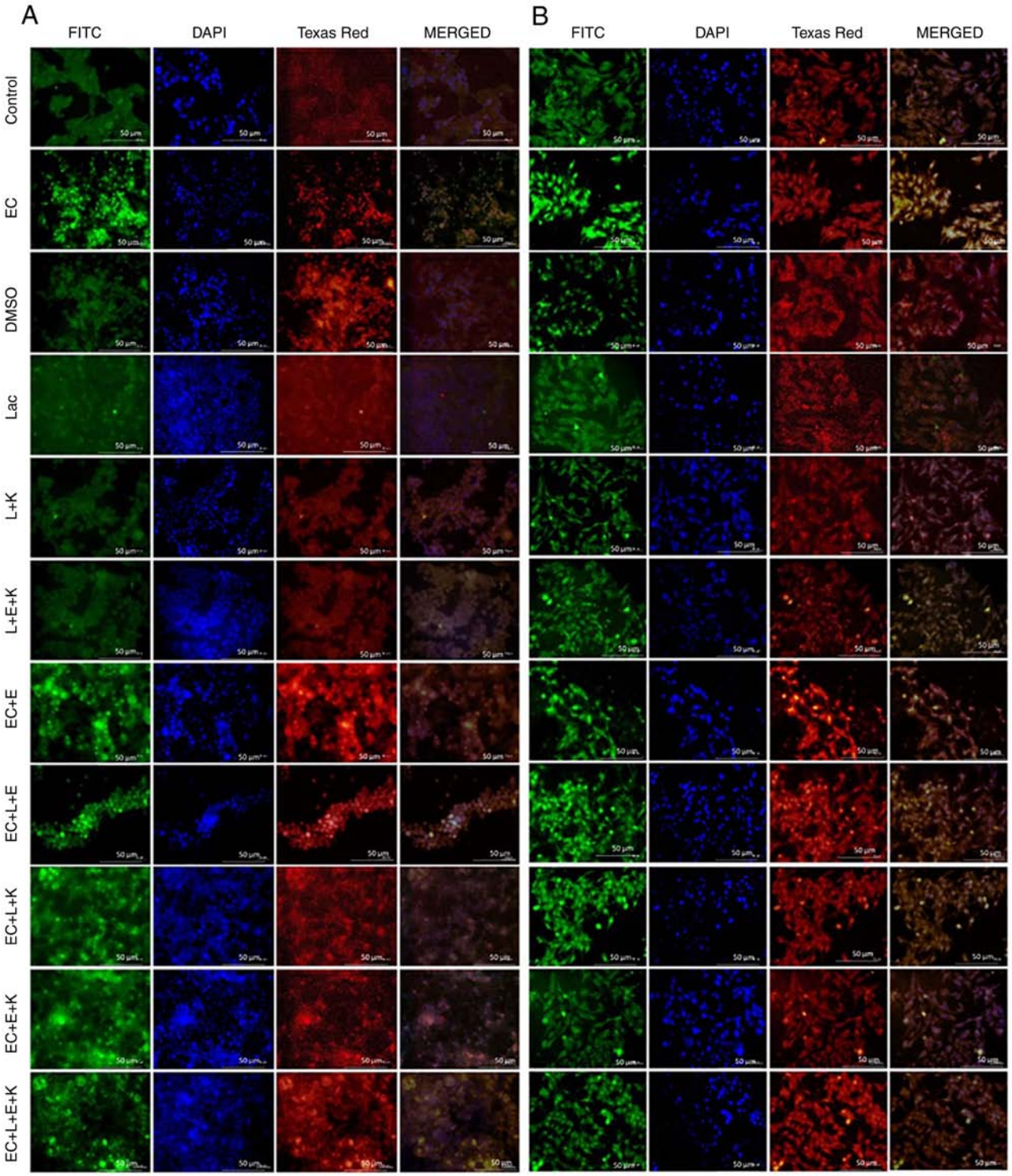

Figure 3. Immunohistochemistry staining. 8-OHdG expression (FITC) and H2A.X expression (Texas Red) in (A) HT-29 and (B) Caco-2 cell lines, immunofluorescence, scale bar, 50 μm. K, vitamin K; E, vitamin E; EC, *Escherichia coli*; L/Lac, *Lactobacillus acidophilus*.

staining showed strong 8-OHdG and H2A.X expressions (Fig. 3). In the DMSO control group, the immunofluorescent staining revealed negative 8-OHdG and H2A.X expressions. The *Lactobacillus* control group developed negative 8-OHdG and H2A.X expressions while the L + vitamin K 1.25 mg/ml group was evaluated as negative 8-OHdG and H2A.X expressions. The L + vitamin E + vitamin K 0.01 group also showed negative 8-OHdG and H2A.X expressions. For the *E. coli* + vitamin E 0.03 group, moderate expressions of 8-OHdG and H2A.X were detected in immunofluorescent staining, while for the *E. coli* + L + vitamin E 0.03 group, mild expressions of 8-OHdG and H2A.X were registered. *E. coli* + L + vitamin K 2.5 mg/ml group: moderate expressions of 8-OHdG and H2A.X detected. For the *E. coli* + vitamin E + vitamin K 0.02 group, only mild expressions of 8-OHdG and H2A.X were noted and for the *E. coli* + L + vitamin E + vitamin K group, low expressions of 8-OHdG and H2A.X were registered in the immunofluorescent staining (Table III).

Table III. Immunofluorescent result scores of the study groups.

	HT-29 8-OHdG	HT-29 H2A.X	Caco-2 8-OHdG	Caco-2 H2A.X
Control	38.85±6.78[a]	21.16±7.18[a]	34.16±6.78[a]	23.76±5.79[a]
E. coli Control	161.22±12.86[b]	96.15±11.83[b]	182.18±10.09[b]	124.28±10.74[b]
DMSO Control	38.15±5.76[a]	23.84±5.12[a]	37.18±5.42[a]	25.18±6.18[a]
Lactobacillus Control	40.12±6.18[a]	25.18±5.6[a]	39.76±6.94[a]	24.74±3.26[a]
Lactobacillus + vitamin K 1.25	41.18±6.23[a]	25.71±6.84[a]	38.12±5.84[a]	22.16±9.12[a]
Lactobacillus + vitamin E + vitamin K 0.01	40.16±6.84[a]	24.6±5.76[a]	36.15±4.12[a]	22.18±5.74[a]
E. coli + vitamin E 0.03	93.18±10.82[c]	78.16±6.12[c]	142.18±10.26[c]	92.18±6.29[c]
E. coli + *Lactobacillus* + vitamin E 0.03	66.85±10.63[d]	50.24±11.38[d]	94.18±14.13[d]	60.12±9.26[d]
E. coli + *Lactobacillus* + vitamin K 2.5	96.18±9.54[c]	73.75±7.76[c]	139.26±9.15[c]	89.26±7.15[c]
E. coli + vitamin E + vitamin K 0.02	68.12±13.79[d]	47.16±9.91[d]	99.16±16.85[d]	61.74±12.7[d]
E. coli + *Lactobacillus* + vitamin E + vitamin K	57.96±11.12[d]	39.97±11.9[d]	85.26±16.28[d]	52.42±10.28[d]

a, b, c and d are differences between means, different letters in the same column are significant (P<0.05).

Table IV. Scoring of immunofluorescent findings in cell cultures.

Group	HT-29 8-OHdG	HT-29 H2A.X	Caco-2 8-OHdG	Caco-2 H2A.X
Control	37.18±6.18[a]	26.14±5.15[a]	39.26±3.12[a]	25.14±6.82[a]
MRSA control	136.14±10.08[b]	108.75±10.84[b]	188.76±13.25[b]	131.18±9.26[b]
DMSO control	39.26±5.81[a]	25.24±4.94[a]	40.12±5.42[a]	28.74±7.59[a]
Lactobacillus control	40.12±4.16[a]	27.18±8.62[a]	41.15±5.8[a]	29.78±4.49[a]
Lactobacillus + vitamin K 1.25	40.16±6.79[a]	28.15±5.54[a]	39.49±4.19[a]	27.65±3[a]
Lactobacillus + vitamin E + vitamin K 0.01	40.74±5.92[a]	27.99±4.84[a]	40.33±6.74[a]	29.48±5.74[a]
MRSA + vitamin E 1.25	91.18±9,74[c]	74.12±6.18[c]	141.16±7.2[c]	102.12±8.18[c]
MRSA + vitamin K 2.5	88.75±8.4[c]	80.16±10.21[c]	137.75±6.74[c]	96.12±9[c]
MRSA + *Lactobacillus* + vitamin E 1.25	72.16±13.26[d]	54.12±11.25[d]	99.15±13.42[d]	68.28±13.26[d]
MRSA + vitamin E + vitamin K 0.007	71.84±10.3[d]	52.28±8.12[d]	96.84±14.35[d]	68.15±12.64[d]
MRSA+ *Lactobacillus* + vitamin E + vitamin K 0.15	64.14±10.98[d]	46.85±10.29[d]	89.4±12.29[d]	57.37±12.94[d]

a, b, c and d are differences between means, different letters in the same column are significant (P<0.05). MRSA, methicillin-resistant *Staphylococcus aureus*.

For the control group, when the HT-29 cell line samples were stained with the double immunofluorescence method, 8-OHdG, and H2A.X expression were evaluated as negative. For the MRSA control group when HT-29 cell line samples were stained with the double-immunofluorescence method, intense 8-OHdG, and H2A.X expressions were observed. In the DMSO control group, negative 8-OHdG and H2A.X expression levels were determined., for the *Lactobacillus* control group negative 8-OHdG and H2A.X expressions were observed, for the L + vitamin K 1.25 group 8-OHdG, and H2A.X were negative, while for L + vitamin E + vitamin K 0.01 group negative 8-OHdG and H2A.X expression levels were determined. In the MRSA + vitamin E 1.25 mg/ml group, when HT-29 cell line samples were stained with the double immunofluorescence method, moderate levels of 8-OHdG and H2A.X was detected. The same results were obtained for the MRSA + vitamin K 2.5 mg/ml group, namely moderate 8-OHdG and H2A.X expressions. Mild 8-OHdG and H2A.X expression levels were determined for the MRSA+L+ vitamin E 1.25 mg/ml and MRSA+ vitamin E + vitamin K 0.007 groups, while for the MRSA + L + vitamin E + vitamin K 0.15 group low levels of 8-OHdG and H2A.X was detected (Fig. 4). The immunofluorescence results are shown in Table IV.

Discussion

There are intense scientific discussions on the effects of probiotics and other supplements such as vitamins. The present study examined how probiotic and vitamin supplementation in HT-29 and Caco-2 colon cancer cells exposed to *Escherichia coli* and MRSA infection would affect the toxicity and viability of the cells. It compared two different colon cancer cells. While HT-29 consists of first-degree cancer cells, Caco-2 cells consist of second-degree cancer cells. By comparing the two levels, the effect of probiotics and vitamin supplementation on the viability of cells in an infection due to the progression of

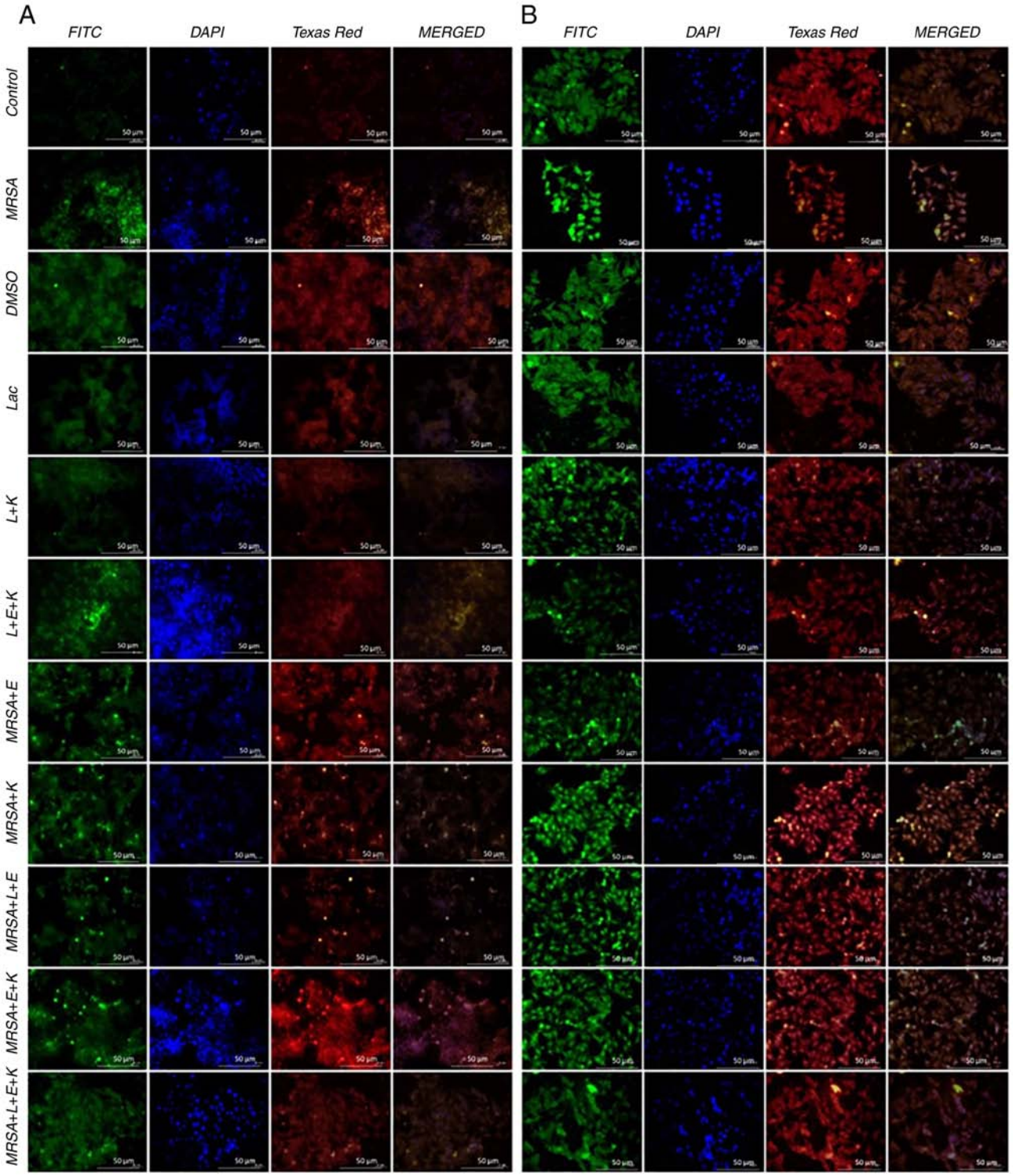

Figure 4. 8-OHdG expression (FITC) and H2A.X expression (Texas Red) in (A) HT-29 and (B) Caco-2 cell lines, immunofluorescence, scale bar, 50 μm. K, vitamin K; E, vitamin E; EC, *Escherichia coli*; L/Lac, *Lactobacillus acidophilus*.

cancer prognosis was examined. Although *Escherichia coli* normally settle in the intestine as avirulent, they acquire virulent characteristics that give them the ability to adapt to new niches and cause intestinal and extra-intestinal diseases. This situation is closely related to the immune system of the host. With these virulent features, they can manage a process ranging from colitis to diarrhea, cancer formation, and mortality (34). In the management of this process, non-antibiotic treatments have gained popularity in recent years with the emergence and spread of new antibiotic-resistant isolates. Among these treatment approaches, the use of probiotics is a promising alternative for the control of urinary tract infections and infections caused by *Escherichia coli*.

Probiotics can adhere to uroepithelial cells and inhibit the growth of pathogenic bacteria. In addition, oral administration of Lactobacilli may colonize these microorganisms in

the urinary tract after intestinal colonization (35). Following a *Staphylococcus aureus* infection, alpha-toxin reaches the basolateral intestinal epithelium. The alpha-toxin then causes barrier malfunction and, as a result, the intestinal lumen becomes permeable to bacteria. As a result, bacteria and bacterial products may pass into the lymph, lymph nodes and, finally, the blood, aggravating the resulting septic condition (6). In the present study, the antibacterial activity of *Lactobacillus acidophilus* and vitamins E and K against *Escherichia coli* was evaluated *in vitro*. The MIC value of vitamin E + vitamin K + *Lactobacillus acidophilus* + MRSA was determined to be 0.15 mg/ml. Ghane *et al* (36) in their study to determine the probiotic potential of *Lactobacillus* strains isolated from kefir and to evaluate their antimicrobial and antibiofilm activities against uropathogenic *Escherichia coli*, screened 12 *lactobacillus* strains for their antimicrobial potentials against uropathogenic *Escherichia coli* and seven of them were isolated from *Escherichia coli* and *Lactobacillus* strains showed high antagonistic activity. Different strains of probiotics are known to produce compounds with antimicrobial properties, including low molecular weight compounds, antimicrobial peptides (bacteriocins) and organic acids (37-41). The coaggregation between Lactobacilli and pathogenic bacteria provides a barrier that prevents them from adhering to urinary and intestinal epithelial cells. In parallel with the present study, Ghane *et al* (36) isolated probiotics in the study that showed antibacterial activity by interacting with *Escherichia coli*.

The probiotic potential of three *Pediococcus* spp. were investigated and 16S rRNA gene sequencing identified *Pediococcus acidilactici* VKU2, *P. acidilactici* IAH-5, and *P. pentosaceus* DHR005. All strains tolerated pH 3 and 0.3% oxalate and simulated gastric and intestinal juice for 3 h (42). *P. acidilactici* IAH-5 showed the highest cholesterol removal (67.52%), hydroxyl radical scavenging activity (58.32%), hydrophobicity (40.3%), and autoaggregation (48%). It was determined that it inhibited the growth of the tested pathogens (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* PTCC 1707, *Salmonella typhimurium* PTCC 1609, and *Staphylococcus aureus* ATCC 25923) and the most susceptible strain was *Staphylococcus aureus* (42). In the present study, the *Lactobacillus acidophilus* strain showed a positive effect against infection in HT-29 and Caco-2 cell lines.

Probiotics are live microorganisms that, when administered in adequate amounts, support health functions in the human or animal host (36). In addition, although various criteria have been proposed for selecting probiotics, the most important feature is their ability to bind to intestinal cells. Due to this adhesion ability, probiotic strains can perform their functions by increasing their persistence in the intestine (36). Due to the difficulty of studying the *in vivo* adhesion of bacteria to the gastrointestinal tract, *in vitro* evaluation using the adenocarcinoma cell lines HT-29 and Caco-2 expresses the morphological and functional features of normal enterocytes, is widely accepted (43). Although most studies on colon cell lines show a correlation between hydrophobicity and adhesion, Schillinger *et al* (44) reported that *Lactobacillus acidophilus* BFE 719 showed good adhesion to HT-29 cells while having a weak hydrophobicity of only 2%. In Lim and Ahn (2012) (45) seven strains isolated from mustard leaf kimchi were screened for their tolerance to simulated gastric and bile juices, their adhesive properties to Caco-2 cells, and their ability to inhibit *Salmonella typhimurium* ATCC 29631 adhesion. *Lactobacillus acidophilus* GK20, *Lactobacillus paracasei* GK74 and *Lactobacillus plantarum* GK81, which are resistant to bile and gastric juices, have been found to have high bile salt hydrolase activity against both sodium glycolate and sodium taurocholate (45). One of the most important features of probiotic bacteria is their ability to survive in severe gastrointestinal conditions, including low pH (i.e., stomach conditions) and high bile salt concentration (i.e., in the small intestine) until reaching their target (46,47). The protection of the intestinal environment by Lactobacilli strains is achieved by two mechanisms: the production of antimicrobial compounds by Lactobacilli and binding to mucus and coaggregation, which can form a barrier to prevent pathogenic biofilm (48). The gut microbiome is associated with the development of colorectal cancer (CRC). Intestinal microbiota and bacterial mass population can trigger the development of CRC by providing the formation of oncometabolite. In a healthy colon, the main part of microbial metabolism is the saccharolytic fermentation pathways (49). It has been suggested that oncogenic bacteria such as Enterotoxigenic *Bacteroides fragilis* induce the development of CRC through direct interactions with colon epithelial cells and changes in microbiota composition in the colorectal region. *Escherichia coli, E. faecalis, Fusobacterium nucleatum*, and *Streptococcus gallolyticus* have been identified as flora with higher populations in CRC patients. However, it has been determined that there is a decrease in the population of *Bifidobacterium*, *Clostridium*, Faecalibacterium, *Lactobacillus,* and *Roseburia* (50). Probiotics such as *Lactobacillus* inhibit the growth of CRC by inhibiting inflammation and angiogenesis and enhancing intestinal barrier function through the secretion of short-chain fatty acids (51). Direct interactions with bacteria and cancer cell combinations and vitamin combinations have not been investigated. From the data of the present study, the toxicity of *E. coli* is higher than that of *lactobacillus* bacteria (52). This is due to the toxins produced by *E. coli*. Cellular death occurs due to the oxidative stress produced by the toxins within the cell. Vitamin E is known to have anti-cancer effects (53). Vitamin E is known to allow potentially beneficial *Lactococcus* and *Bacteroides* to multiply in the gut (53). In a colitis-associated cancer model, vitamin E suppresses proinflammatory cytokines and modulates the gut microbiota (54). The combination of two bacteria and two vitamins in the present study did not cause damage to the bacterial mass by reducing the neoplastic cell population (55).

In recent years, microbiome methods have been used in fecal collection using next-generation sequencing technology and DNA analysis of all bacteria. The present study performed no experiments on humans Future studies could experimentally administer the probiotics, vitamin K and vitamin E. used here for healthy subjects. and attempting to analyze their gut microbiota. Furthermore, epidemiological studies linking the consumption of these products in the human population would be another strategy.

Probiotics, an alternative treatment option for controlling infections caused by *Escherichia coli* and MRSA, prevent the growth of pathogenic bacteria and this can be helped by additional vitamin support. In this context, increasing additional support studies for probiotic treatment are vital for the course of treatment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

DC and AlT conceived the present study. ArT, OC, DAS and DC were responsible for the methodology. ALA, RM and KG were responsible for the formal analysis. AlT and RM performed investigations. ArT, DAS, and AlT were responsible for resources. ALA, KG and AlT wrote the original draft. ArT, OC, DC, RM and ALA reviewed and edited the manuscript. KG and ALA were responsible for visualization. ArT and AlT were responsible for supervision. DC and AlT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. DS is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article.

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