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# Inactivation of foodborne pathogenic and spoilage microorganisms using ultraviolet-A light in combination with ferulic Acid

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Running headline: Synergistic antimicrobial activity

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## SIGNIFICANCE AND IMPACT OF THE STUDY (100 words)

Microbial contamination is one of the most serious problems for foods, fruit and sugar thick juices. UV light is suitable for non-thermal decontamination of food products by inactivation of contaminating microorganisms. However, UV-A exposure is insufficient for disinfection. This study demonstrates that the combination of UV-A LED light (350-385 nm), which is not hazardous to human eyes and skin, and ferulic acid (FA), a known phytochemical and food additive, provides synergistic antimicrobial activity against foodborne pathogenic and spoilage microorganisms. Therefore, FA addition to UV-A light treatment may be useful for improvement of UV-A disinfection technology to prevent food deterioration.

## ABSTRACT (200 WORDS)

The low energy of UV-A (315–400 nm) is insufficient for disinfection. To improve UV-A disinfection technology, the effect of ferulic acid (FA) addition on inactivation by UV-A light-emitting diode (LED) light (350–385 nm) was evaluated in the eliminating of suspensions of various food spoilers and pathogens (seven bacteria and four fungi). Photoantimicrobial assays were performed at FA concentrations below the MIC. The MIC of the isomerized FA, consisting of 93% *cis*-form and 7% *trans*-form, was very similar to that of the commercially available FA (*trans*-form). Irradiation with UV-A (1.0 J $cm^{-2}$) in the presence of 100 mg $l^{-1}$ FA resulted in enhanced reducing of all of the tested bacterial strains. A combination of UV-A (10 J $cm^{-2}$) and 1000 mg $l^{-1}$ FA resulted in enhanced reducing of *Saccharomyces cerevisiae* and one of the tested filamentous fungi. These results demonstrated that the combination of a short-term application of UV-A and FA at a low concentration yielded synergistic enhancement of antimicrobial activity, especially against bacteria.

**Keywords**: ferulic acid, photoantimicrobial activity, UV-A, food spoilers, foodborne pathogens

## Introduction

UV-bactericidal technology, which produces no residual chemicals and has little influence on the environment, is a convenient method for disinfection of gases, liquids, and solid surfaces. In recent years, a versatile UV source has been provided by the development of light-emitting diodes (LEDs), which yield constant illumination at a specific wavelength and do not contain mercury. LEDs are also advantageous because of their durability and low heat generation (Shin *et al.* 2016). A LED illumining UV-C light (266 nm or 275 nm), which is defined as radiation with wavelengths 200–280 nm and has traditionally been used as an effective germicidal disinfectant, exhibited high bactericidal activity (Kim *et al.* 2016; Shin *et al.* 2016). UV-C LED irradiation (266 nm; irradiance 4 μW $cm^{-2}$) reduced the viability of *Escherichia coli* O157:H7 by 6 log-units at 0.5 mJ $cm^{-2}$. UV-A (315–400 nm) has been also reported to exhibit photobactericidal activity (Hamamoto *et al.* 2007). Notably, exposure to UV-A is not hazardous to human eyes and skin; in contrast, UV-B and UV-C light (i.e. at wavelengths <315 nm) can induce sunburn, cell mutations, and cell death (Gruijl 2002). Moreover, in the food industry, UV-C exposure is known to inactivate polyphenolic acids (chlorogenic acid and phloridzin) that are abundant in apple juice, thus decreasing the antioxidative activity of this product (Islam *et al.* 2016). These findings suggest that UV-A light may be suitable for non-thermal decontamination of food products by inactivation of contaminating microorganisms. However, UV-A light, being of lower energy than UV-C, exhibits lower photobactericidal activity. To completely inactivate *E. coli* using UV-A LED irradiation alone

(365 nm), a 315-J $cm^{-2}$ fluence (at an irradiance of 70 mW $cm^{-2}$ for 75 min) was required (Hamamoto *et al.* 2007).

To increase the inactivation efficiency of UV-A, the synergistic bactericidal activity of the combination of UV-A light and organic substrates, a quaternary ammonium salt (Shirai et al. 2014), and natural compounds [coumaric acid and ferulic acid (FA)] and their derivatives(Shirai et al. 2015a, 2015b), has been investigated in our laboratory. In those reports, the addition of FA enhanced the photobactericidal activity of the UV-A such that the combination of approximately 20 mg $l^{-1}$ FA with UV-A (irradiance 4.09 mW $cm^{-2}$, 30 min) resulted in a >5-log decrease in the survival of *E. coli*.

FA, one of the most abundant phenolic acids in plants such as rice, wheat, barley, citrus fruits, and tomatoes, occurs as esters conjugated covalently with mono-, di-, and polysaccharides and with lignin of cell walls (Graf 1992; Paiva *et al.* 2013). It is a potential therapeutic agent with demonstrated antioxidant, antimicrobial, hepatoprotective, and UV protective activities (D Paiva *et al.* 2013). In Japan, FA is approved for use as an antioxidant food additive (JFCRF 1996).

Microbial contamination is one of the most serious problems for foods, fruit and sugar thick juices (Braun *et al.* 1999; Fleet 2007; Juste *et al.* 2008; Tribst *et al.* 2009). In those reports, *Salmonella* are typical pathogenic bacteria associated with fruit juices. *Alicyclobacillus* and *Sporolactobacillus* have been isolated from spoiled fruit juices and can survive heat treatments by forming heat-tolerant spores. Yeasts affect negatively juice's flavor, turbidity and odor. The microorganisms that we tested included two heat-tolerant filamentous fungi (*Byssochlamys fulva* and *Eupenicillium lapidosum*) and a third filamentous fungus, *Cladosporium cladosporioides*, found in canned or bottled fruit. *E. coli* is commonly used as

an indicator organism. As an UV-A disinfection technology for applications in the food industry, the goal of this study was to investigate the synergistic effect of FA on the photoantimicrobial activity of UV-A (wavelength range 350–385 nm produced by a LED source) using those multiple food spoilers and pathogens and to reveal enhancement of photoantimicrobial activity by FA addition.

## Results and Discussion

### Antimicrobial activity of FA isomers

FA (*trans*-form) and isomerized FA, consisting of 93% *cis*-form and 7% *trans*-form, were evaluated for antimicrobial activity on the basis of MIC assays against various microorganisms (Table 1). The MICs for both FAs were 125 to 1000 mg $l^{-1}$ or >1000 mg $l^{-1}$ for the tested microorganisms. The MICs of isomerized FA and *trans*-FA were very similar and no significant differences were observed between the isomers.

>Table 1<

Other work has shown that *cis*-form phenyl compounds substituted with propenoic acid moieties have notable biological properties. For instance, the antimicrobial activity and the anti-invasive activity (against adenocarcinoma cells) of cinnamic acid depend on its isomerization (Chen *et al.* 2011; Yen *et al.* 2011). The *cis*-isomer of cinnamic acid has a strong bactericidal effect against a multidrug-resistant *Mycobacterium tuberculosis* at a concentration two orders of magnitude lower than that observed for the *trans*-isomer (Chen *et al.* 2011). Cinnamic acid acts by causing a disruption in the cell wall of the bacterium, resulting in a wrinkled and rough colony phenotype in micrographs. Similarly, other phenolic acid compounds (caffeic acid and gallic acid) have been shown to bind to (or be incorporated into)

the cytoplasmic membrane of *E. coli* and *S. aureus* (Nakamura *et al.* 2015). FA is isomerized from the *trans*-form to the *cis*-form through phenoxy radical formation during UV exposure (Graf 1992). Photoisomerization of FA to the *cis*-isomer could directly injure the cell membrane (as *cis*-cinnamic acid does). By analogy to cinnamic acid, we expected that the antimicrobial activity of FA would exhibit isomeric specificity, such that the inhibitory activity of the *cis*-form of FA would be much higher than that of the *trans*-form. However, the MICs of isomerized FA and *trans*-FA were very similar in our experiments, suggesting that the antimicrobial activity of FA does not depend on isomerization. Therefore, the photo-induced conversion of FA would have no effect on its photoantimicrobial activity when combined with UV-A irradiation.

**Photoantimicrobial activity**

Photoantimicrobial assays were performed at FA concentrations below its MIC. Bactericidal activity against *E. coli* was investigated by treating this organism with a combination of FA and UV-A irradiation. A total UV-A fluence at 30 J $cm^{-2}$ (58.3-min irradiation) in the absence of FA resulted in a 2.82-log unit reduction in the viable cell count [Fig. 1(A)]. The addition of FA to the suspension before UV-A irradiation enhanced bactericidal activity in a manner that was dependent on FA dose and UV-A fluence. The combination of FA (at 50, 100, and 150 mg $l^{-1}$) with UV-A light [at 1.5 J $cm^{-2}$ (2.92-min irradiation), 1.0 J $cm^{-2}$ (1.95-min irradiation), and 0.75 J $cm^{-2}$ (1.46-min irradiation), respectively] yielded viable cell counts of <10 CFU $ml^{-1}$ [Fig. 1(B)]. Compared with the bactericidal activity of UV-A at 3.0 J $cm^{-2}$ fluence in the absence of FA, the combination of FA with UV-A yielded significant ($P < 0.01$) decreases in the viable cell count. Exposure to 150 mg $l^{-1}$ FA for an equivalent time interval (5.83 min, the incubation time used above to provide a fluence of 3.0 J $cm^{-2}$ UV-A) in the absence of UV-A

(i.e., in the dark) had a much smaller antibacterial effect (0.37-log reduction in *E. coli* cell density).

>Figure 1<

The efficacy of the combination of FA (100 mg $l^{-1}$) and UV-A (1.0 J $cm^{-2}$) was tested with six other bacterial strains (Fig. 2). Under these conditions, cell counts of *Salm. enterica*, *Staph. aureus*, and *Sporolact. inulinus* decreased below the detection limit of 10 CFU $ml^{-1}$. Cell counts of *B. cereus*, *K. rhizophila* and *A. acidoterrestris* decreased by 4.04-logs, 3.75-logs and 2.20-logs, respectively. In contrast, no significant decrease in viability was observed for any of these strains (except for *Sporolact. inulinus*) when exposed to FA in the absence of UV-A (for 1.95 min, the incubation time used above to provide a fluence of 1.0 J $cm^{-2}$ UV-A), or to the UV-A fluence in the absence of FA. For *Sporolact. inulinus*, a decrease in viable cells (3.15-logs) was observed upon treatment with FA in the dark. Notably, the combined treatments with UV-A and FA provided significant ($P < 0.01$) increases in bactericidal activity compared with those with FA addition alone or UV-A irradiation alone.

>Figure 2<

In other work, Nakamura et al. (2015) showed that irradiation with short wavelength light (380–420 nm; irradiance 0.26 W $cm^{-2}$) in combination with caffeic acid (a polyphenol similar to FA) killed each of four bacterial strains; notably, however, these effects required a fluence of 78 J $cm^{-2}$ and a caffeic acid concentration of 1000 mg $l^{-1}$. In contrast, inactivation of bacteria by the methods described in the present study required much lower light fluence and reagent concentration. Potent inactivation (to microbial densities below 10 CFU $ml^{-1}$, the lower limit of detection) of *E. coli* was achieved at total fluences of 1.0 J $cm^{-2}$ in combination with a FA concentration of 100 mg $l^{-1}$. Similar enhancement of UV-A bactericidal efficacy

was also demonstrated for the other six tested bacterial strains. The bactericidal synergy of UV-A and FA against some Gram-positive bacteria, including some *Bacillus*, *Kocuria* and *Alicyclobacillus*, was lower than against Gram-negative bacteria. As shown in a previous report by Nakamura et al. (2015), the affinity of FA, which is an analog of caffeic acid, to Gram-positive bacteria should be lower than to Gram-negative bacteria. These results demonstrated that the combination of UV-A and FA yielded synergistic enhancement of bactericidal activity while using a short-term application of UV-A light (1.0 J $cm^{-2}$) in combination with 100 mg $l^{-1}$ FA. We infer that the high synergism of UV-A light with FA reflects the production of phenoxy radicals that in turn leads to the production of hydrogen peroxide as supported by the fact that photobactericidal activity is quenched by the addition of catalase (Shirai *et al.* 2015b). In this work, microbial inactivation by the FA + UV-A regime was assayed against vegetative cells. Future work on the synergistic efficacy will be needed to investigate activity against spores of organisms like *B. subtilis*, *B. cereus*, *A. acidoterrestris* and *Sporolact. inulinus*.

We investigated antifungal activity by testing the effect of the combination of FA (1000 mg $l^{-1}$) and UV-A (10 J $cm^{-2}$ fluence) on *S. cerevisiae* and three filamentous fungi.

UV-A irradiation alone, at fluences of up to 10 J $cm^{-2}$, yielded little decrease in viable yeast cell counts (Fig. 3). Exposure to 1000 mg $l^{-1}$ FA for an equivalent time interval (19.4 min, the incubation time used above to provide a fluence of 10 J $cm^{-1}$ UV-A) in the absence of UV-A (i.e., in the dark) resulted in a very small amount of anti-yeast activity (0.05-log reduction in yeast cell density). The anti-yeast activity of UV-A was enhanced in the presence of 1000 mg $l^{-1}$ FA; at a fluence of 10 J $cm^{-2}$ in the presence of 1000 mg $l^{-1}$ FA, yeast viability fell below the detection limit of 10 CFU $ml^{-1}$.

>Figure 3<

The photoantifungal activity against *B. fulva* was significantly increased in the presence of FA, with survival ratios without and with FA of 0.01-logs and 1.49-logs, respectively ($P < 0.01$). In contrast, FA addition did not significantly enhance photoinactivation of *C. cladosporioides* by UV-A irradiation ($P > 0.05$), though the treatment yielded a nominal decrease in viability. Similarly, FA addition did not significantly enhance photoinactivation of *E. lapidosum*. For the three filamentous fungi, FA addition alone and UV-A irradiation alone had a low fungicidal activity. Additional investigations with various combinations of irradiance and FA concentration may reveal conditions suitable for the inactivation of those fungi; such efficacy would be of great value, given that these organisms are often resistant to heating and UV-C exposure (254 nm) (Hamanaka *et al.* 2010).

Several laboratories have shown that UV-A LED light exhibits much lower photobactericidal activity (Hamanoto *et al.* 2007; Shirai *et al.* 2014) than UV-C LED light (Kim *et al.* 2016). Inactivation with UV-A light alone requires high irradiation and long exposure times. In the present work, to increase the inactivation efficiency of UV-A, we investigated the FA + UV-A combination with respect to its photoantimicrobial activity against various foodborne pathogenic and spoilage microorganisms related to foods, fruit and sugar thick juices.

In conclusion, a combination of FA with UV-A irradiation resulted in a significant enhancement in their individual efficacy toward the tested organisms. The high synergistic activity against bacteria was observed when UV-A at low fluence and short time exposure was combined with FA at a low concentration. Reducing of yeast and at least one filamentous fungus was observed upon treatment with longer UV-A exposure and higher FA concentration.

Therefore, FA combined with UV-A light treatment may be useful for the improvement of UV-A disinfection technology. Also, in future work, surface decontamination of fruits will be investigated using the combination of UV-A light and FA for applications in postharvest disinfection.

## Materials and Methods

### Microbial strains

Microorganisms used in the determination of antimicrobial activity were purchased from NITE Biological Resource Center (NBRC), American Type Culture Collection (ATCC) and Institute of Food Microbiology (IFM). Those strains are listed in Table 1. Endospore-forming bacteria (*Bacillus*, *A. acidoterrestris* and *Sporolact. inulinus*) were tested in their vegetative state.

### UV-A source and irradiation.

A device equipped with a UV-A LED (NCSU033B; Nichia Corp., Anan, Japan), as previously described, was used in all photoexperiments (Shirai *et al.* 2015b). The LED has a radiation angle of about 120° as the full width at half maximum. The peak wavelength was 365 nm and the wavelength range was 350–385 nm, which was measured with a cumulative UV meter (MCPD-3700A; Otsuka Electronics Co. Ltd., Hirakata, Japan) (Fig. S1).

### Chemistry

FA (PubChem CID: 445858) was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Except as noted, FA used for testing consisted of the *trans*-form obtained as commercially available FA.

### MIC assay

Details regarding preparation, separation and purity of isomerized FA are summarized in the Supporting Information (Text S1). The antimicrobial activity of FA and the isomerized FA, consisting of 93% *cis*-form and 7% *trans*-form, against the organisms listed above was evaluated by determining MICs using the broth dilution method (Shirai *et al.* 2005). Preculture of organisms was performed with the conditions described in Table S1. Cell density was determined from $OD_{660}$ using a UV-1700 spectrophotometer (Shimadzu Ltd., Kyoto, Japan) for bacteria and yeast, and using a hemocytometer (Burker-Turk; depth 0.1 mm, 1/400 qmm; Erma Inc., Tokyo, Japan) for filamentous fungi.

FA solutions for testing against bacteria (except *A. acidoterrestris* and *Sporolact. inulinus*) were generated by diluting the FA stock solution [100 g $l^{-1}$ in 80% dimethylsulfoxide (DMSO)] with nutrient broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) to generate the highest testing concentration of 1000 mg $l^{-1}$; this solution was then subjected to a two-fold serial dilution using nutrient broth. To generate FA solutions for testing against *A. acidoterrestris* and *Sporolact. inulinus*, all dilutions (starting from the stock solution) were performed as above but using a specific broth (No. 323), which is recommended on the NBRC website (NBRC 2016), and GYP broth [glucose 2% (w/v), yeast extract 0.5% (w/v), Bacto peptone 0.5% (w/v)] (Kitahara and Suzuki 1963), respectively. To generate FA solutions for testing against fungi, all dilutions (starting from the stock solution) were performed as above but using Sabouraud broth [polypeptone 1% (w/v) and glucose 4% (w/v)]. The final cell densities were approx. $1\times10^5$ CFU $ml^{-1}$ for bacteria and yeast, and approx. $1\times10^4$ conidia $ml^{-1}$ for filamentous fungi in a transparent 96-well culture plate (Corning Inc., NY, USA). MICs for *A. acidoterrestris* and *Sporolact. inulinus* were determined after 24-h incubation at 45°C and 48-h incubation at 37°C in an anaerobic chamber with an AnaeroPack Kenki that can reduce

the oxygen percentage to <0.1% within 2 h (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan), respectively. MICs for the other bacteria, yeast, and filamentous fungi were determined after 24-h incubation in ambient air at 37°C, 28°C, and 25°C, respectively.

**Photoantimicrobial assay**

Photoantimicrobial activity against bacteria and fungi was determined by plating and counting the colony-forming units remaining after treatment of a microbial suspension as reported previously (Shirai *et al.* 2014). Cells of organisms, precultured according to Table S1, were prepared as described in the same report. Conidial suspensions were prepared as described previously (Shirai *et al.* 2005). Cell density was determined as described in the section above.

All antimicrobial assays with UV-A irradiation or with no irradiation (in the dark) were performed in an incubator box maintained at 30°C, using suspensions of organisms placed in the individual wells of a transparent 48-well culture plate (AGC Tecno Glass Co. Ltd., Tokyo, Japan). An aliquot of microbial suspension (0.1 ml, approx. $2\times10^7$ CFU $ml^{-1}$ for bacteria or approx. $2\times10^5$ CFU $ml^{-1}$ for fungi) was added to each well of a 48-well culture plate already containing 0.01 ml of test compound at concentrations of 5, 10, 15, and 100 g $l^{-1}$ in 80% DMSO and 0.89 ml of sterile water per well (volume of the final tested suspensions 1 ml; depth 13 mm). For UV-A irradiation alone (no test compound), DMSO was added to a concentration of 0.8% to each well of the 48-well plate. The single UV-A LED was placed face-up to permit upward irradiation into the bottom of the 48-well culture plate. The device was set 30 mm (height) from the middle of the tested suspensions. The intensity of was 8.58 mW $cm^{-2}$ at the bottom of the well, which was measured with a laser power and energy meter (Nova II; Ophir Optronics Solutions Ltd., Saitama, Japan) equipped with a photodiode sensor (PD-300-UV; Ophir Optronics Solutions Ltd.). Total fluence tested (0.5, 0.75, 1.0, 1.5, 3.0, 10,

and 30 J $cm^{-2}$) was calculated based on the irradiance (8.58 mW $cm^{-2}$) and exposure time (0.97 to 58.3 min). After treatments with or without UV-A irradiation for bacteria, aliquots (0.15 ml) of each suspension were diluted 10-fold with SCDLP broth (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) followed by serial 10-fold dilutions with 0.8% (w/v) physiological saline containing 0.7% (w/w) Tween 80 (Kanto Chemical Co., Inc., Tokyo, Japan). Viable cell counts (CFU $ml^{-1}$) after 10-fold serial dilutions were determined by plating on suitable agar plates and incubating them (Table S1). For fungi, 10-fold serial dilutions were performed with Sabouraud broth containing 0.1% (w/w) Tween 80.

Antimicrobial activity was expressed as the log survival ratio (log S) according to the equation: $\log S = \log(N_t/N_0)$, where $N_0$ represents the number of CFUs before bactericidal treatment and $N_t$ represents the number of CFUs after treatment for time $t$.

**Statistical analysis**

All antimicrobial experiments were performed as three independent procedures, and results are presented as the mean and SD. Inferential analysis was performed using a two-tailed, unpaired Student's $t$-test. $P$-values of $<0.05$ were considered significant.

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**Conflict of Interest**

No conflict of interest declared.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Text S1.** Preparation, separation, and purity of *cis*-FA.

**Table S1.** Conditions for preculture and viability assay of microorganisms tested.

**Figure S1.** Emission spectrum of the UV-A LED as used in this study; the spectrum exhibited a maximum at 365 nm, which was measured at a distance of 30 mm between the illumination source and the UV meter.

**Figure S2.** HPLC chromatogram of FA prepared by UV-A exposure: before separation (A), and after separation and purification of the *cis*-form (B).

**Figure S3.** UV-visible absorption spectra of FA (*trans*-form, solid line) and isomerized FA (mixture of 93% *cis*-form and 7% *trans*-form, dashed line). Samples were dissolved at 50 μM in 0.4% DMSO.

## FIGURE LEGENDS

**Figure 1.** UV-A fluence-dependent changes in *E. coli* survival following irradiation in the absence (A) or presence (B) of FA. Filled (black) symbols, no UV-A exposure; unfilled (white) symbols, UV-A exposure. Cell suspensions were treated with FA at 50 mg $l^{-1}$ (diamond), 100 mg $l^{-1}$ (triangle), or 150 mg $l^{-1}$ (circle). Data are presented as means ± SD (n = 3). Significant differences (**$P < 0.01$) were calculated based on comparison to samples

irradiated with UV-A (3.0 J $cm^{-2}$) in the absence of FA. Samples for which survival was <10 CFU $ml^{-1}$ (lower limit of detection) are noted in parentheses as 6-log unit reductions.

**Figure 2.** Photobactericidal activity against the six indicated bacterial species of UV-A irradiation (1.0 J $cm^{-2}$) in the absence (light-gray bars) and presence (unshaded bars) of 100 mg $l^{-1}$ FA. Black bars indicate 100 mg $l^{-1}$ FA treatment in the absence of UV-A (for 1.95 min, the incubation time used to provide a fluence of 1.0 J $cm^{-2}$ UV-A). Data are presented as means ± SD (n = 3). Significant differences (**$P < 0.01$) were calculated based on comparison to viability of the respective bacterium exposed to FA alone and to UV-A exposure without FA. Samples for which survival was <10 CFU $ml^{-1}$ (lower limit of detection) are represented as 6-log reductions.

**Figure 3.** Photoantimicrobial activity against four fungal species of UV-A irradiation (10 J $cm^{-2}$) in the absence (light-gray bars) and presence (unshaded bars) of 1000 mg $l^{-1}$ FA. Black bars indicate 1000 mg $l^{-1}$ FA treatment in the absence of UV-A (for 19.4 min, the incubation time used to provide a fluence of 10 J $cm^{-2}$ UV-A). Significant differences (**$P < 0.01$) were calculated based on comparison to viability for the respective fungus exposed to FA addition alone and UV-A without FA. Samples for which survival was <10 CFU $ml^{-1}$ (lower limit of detection) are noted in parentheses as 4-log unit reductions.

394 **Table 1** MICs of *trans*-FA and isomerized FA

| Microorganism | MIC (mg $l^{-1}$)* | |
|---|---|---|
| | *trans*-FA | Isomerized FA** |
| *Escherichia coli* NBRC12713 | >1000 | >1000 |
| *Salmonella enterica* NBRC13245 | 1000 | 1000 |
| *Staphylococcus aureus* NBRC12732 | 500 | 1000 |
| *Bacillus cereus* NBRC15305 | 1000 | 1000 |
| *Bacillus subtilis* ATCC6633 | 1000 | 670 ± 290 |
| *Kocuria rhizophila* NBRC12708 | 500 | 500 |
| *Alicyclobacillus acidoterrestris* NBRC108913 | 125 | 125 |
| *Sporolactobacillus inulinus* NBRC13595 | 500 | 500 |
| *Saccharomyces cerevisiae* NBRC1136 | >1000 | >1000 |
| *Cladosporium cladosporioides* IFM63149 | >1000 | >1000 |
| *Byssochlamys fulva* NBRC31767 | >1000 | >1000 |
| *Eupenicillium lapidosum* NBRC6100 | >1000 | >1000 |

395

396 *Values are the mean ± SD obtained from three independent experiments. Values without SDs
397 were identical in each of the three independent experiments.

398 **Composed of 93% *cis*-form and 7% *trans*-form

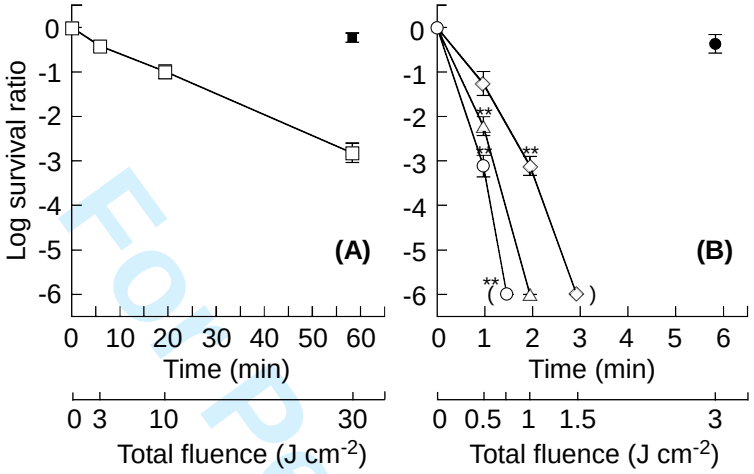


**Fig 1** UV-A fluence-dependent changes in *E. coli* survival following irradiation in the absence (A) or presence (B) of FA. Filled (black) symbols, no UV-A exposure; unfilled (white) symbols, UV-A exposure. Cell suspensions were treated with FA at 50 mg $l^{-1}$ (diamond), 100 mg $l^{-1}$ (triangle), or 150 mg $l^{-1}$ (circle). Data are presented as means ± SD (n = 3). Significant differences (**$P < 0.01$) were calculated based on comparison to samples irradiated with UV-A (3.0 J $cm^{-2}$) in the absence of FA. Samples for which survival was <10 CFU $ml^{-1}$ (lower limit of detection) are noted in parentheses as 6-log unit reductions.

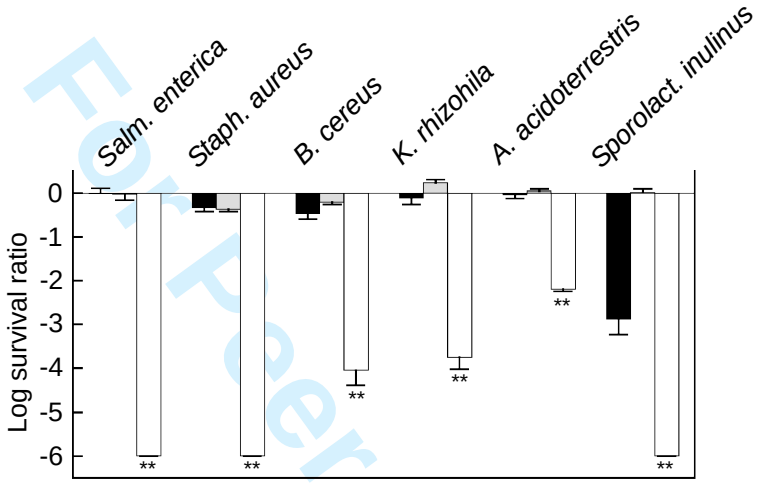


**Fig 2** Photobactericidal activity against the six indicated bacterial species of UV-A irradiation (1.0 J $cm^{-2}$) in the absence (light-gray bars) and presence (unshaded bars) of 100 mg $l^{-1}$ FA. Black bars indicate 100 mg $l^{-1}$ FA treatment in the absence of UV-A (for 1.95 min, the incubation time used to provide a fluence of 1.0 J $cm^{-2}$ UV-A). Data are presented as means ± SD (n = 3). Significant differences (**$P < 0.01$) were calculated based on comparison to viability of the respective bacterium exposed to FA alone and to UV-A exposure without FA. Samples for which survival was <10 CFU $ml^{-1}$ (lower limit of detection) are represented as 6-log reductions.

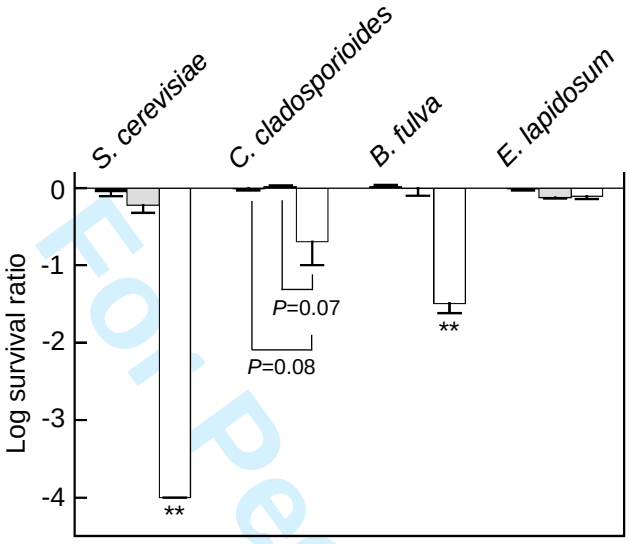


**Fig 3** Photoantimicrobial activity against four fungal species of UV-A irradiation (10 J $cm^{-2}$) in the absence (light-gray bars) and presence (unshaded bars) of 1000 mg $l^{-1}$ FA. Black bars indicate 1000 mg $l^{-1}$ FA treatment in the absence of UV-A (for 19.4 min, the incubation time used to provide a fluence of 10 J $cm^{-2}$ UV-A). Significant differences (**$P < 0.01$) were calculated based on comparison to viability for the respective fungus exposed to FA addition alone and UV-A without FA. Samples for which survival was <10 CFU $ml^{-1}$ (lower limit of detection) are noted in parentheses as 4-log unit reductions.

**Preparation, separation, and purity of *cis*-FA.**

*cis*-FA was prepared by isomerization of commercially available FA by UV-A irradiation. FA (250 mg) was dissolved in 5 ml methyl alcohol (HPLC grade) and the mixture was irradiated using the UV-A LED, as used in the photoantimicrobial assay, at 6.18 mW $cm^{-2}$ for 15 min at room temperature (around 25°C). The LED was used to irradiate downward into a standard glass Petri dish (internal diameter 27 mm) without a cover at a distance of 50 mm between the LED and the bottom of the Petri dish.

Isolation of the *cis*-isomer from the *cis*- and *trans*-FA mixture was performed using a Shimadzu HPLC system (pump, LC-20AT; auto-sampler, SIL-20AC; UV-detector, SPD-M20A; column oven, CTO-20AC; Kyoto, Japan) equipped with a COSMOSIL column (HILIC, 10 × 250 mm; Nacalai Tesque Inc.) using acetonitrile/10 mM ammonium acetate (aq.) (9:1) as the mobile phase (flow rate 8 ml $min^{-1}$). The detection wavelength was 318 nm, which was the maximum absorbance wavelength of the mixture. The resulting HPLC spectrum before separation is shown in Figure S2(A). Two major peaks were detected with retention times of 21–38 min and 39–45 min; these peaks were assigned as *trans*-FA and *cis*-FA, respectively, on the basis of the proton nuclear magnetic resonance ($^1$H-NMR) spectra using a JEM-EX 400 spectrometer (JOEL, Tokyo, Japan).

After the *cis*-FA eluent was collected, the organic solvent was evaporated. The residual solution was adjusted to pH 3–4 with 6 M HCl (aq.). An ethyl acetate layer extracted from the solution was washed with brine (sat. NaCl sol.), dried over sodium sulfate, and concentrated under reduced pressure. Purification by flash chromatography on silica gel (silica gel 120,

mesh 70–230 spherical; Nacalai Tesque Inc.) using elution with chloroform/methyl alcohol (20:1) yielded *cis*-FA (yellowish, oily). Mass analysis (Acquity UPLC-LCT Premier liquid chromatography-mass spectrometry system; Nihon Waters K.K., Tokyo, Japan) yielded a mass for the isomerized FA consistent with the expected value for this compound [ESI-MS (*m/z*) for $C_{10}H_{10}O_4$, calculated: 194.06 $[M]^+$, found: 195.066 $[M+H]^+$].

The proportions of the *cis*- and *trans*-forms in the isomerized FA after separation and purification were determined using the Shimadzu HPLC equipped with a COSMOSIL column (HILIC, 4.6 × 150 mm; Nacalai Tesque Inc.) at a detection wavelength of 318 nm. The elution solvent was acetonitrile/10 mM ammonium acetate (aq.) (8:2) at a flow rate of 0.5 ml $min^{-1}$ [Fig. S2(B)]. To determine the difference in peak area corresponding to *trans*-FA, commercially available *trans*-FA and the isomerized FA were (separately) dissolved at 0.35 mg $ml^{-1}$ in 80% DMSO and assayed. The isomerized FA was shown to be composed of 93% *cis*-form and 7% *trans*-form on the basis of the HPLC analysis. Additionally, the isomerized FA was analyzed with $^1$H-NMR; for this assay, the isomerized FA was dissolved in DMSO-$d_6$ with tetramethylsilane as an internal standard. Integrated values for the α-H of the double bond in the propenoic acid moiety corresponding to *cis*-FA and *trans*-FA were compared. $^1$H-NMR analysis confirmed that the isomerized FA included both isomers, as demonstrated by detection of the α-H of double bonds in each propenoic acid moiety corresponding to *cis*- and *trans*-FA; the coupling constants were 13.0 Hz (δ, 5.73 ppm; doublet peak) and 15.9 Hz (δ, 6.36 ppm; doublet peak), respectively. These NMR data were consistent with those in a previous report (Guo *et al.* 2015). The proportion of the *cis*-form, calculated from the integrated values for the

α-H protons relevant to the *cis*- and *trans*-forms, respectively, was similar to that derived based on HPLC analysis.

For determination of UV-visible absorbance spectra, commercially available *trans*-FA and the isomerized FA were (separately) dissolved at 50 μM in 0.4% (v/v) DMSO. Spectra were measured using a 1-cm pathlength cuvette and a U-3300 spectrophotometer (Hitachi Ltd., Tokyo, Japan). The UV absorption spectrum of the *cis*-form was distinct from that of the *trans*-form (Fig. S3); the difference was very similar to that observed between resveratrol isomers (Trela and Waterhouse 1996).

When the isomerized FA was prepared with 80% DMSO or pure water, the composition was stable at 30°C for at least 98 days, as judged by negligible time-dependent changes in the area of the HPLC peak corresponding to *trans*-FA (data not shown). The isomerized FA, which was highly enriched for the *cis*-form, was used for determination of MICs.

**References**

Guo, J., Zhang, J., Wang, W., Liu, T. and Xin, Z. (2015) Isolation and identification of bound compounds from corn bran and their antioxidant and angiotensin I-converting enzyme inhibitory activities. *Eur Food Res Technol* **241**, 37-47.

Trela, B.C. and Waterhouse, A.L. (1996) Resveratrol: isomeric molar absorptivities and stability. *J Agric Food Chem* **44**, 1253-1257.

**Table S1** Conditions for the preculture and viability assay of the tested microorganisms

| Microorganism | Preculture condition<br>Medium / Temp. / Time of growth | Viability assay<br>Medium / Temp. / Time of growth |
|---|---|---|
| *E. coli* NBRC12713 | LB* / 37°C / 17 h with shaking | SCDLP agar†† / 37°C / 48 h |
| *Salm. enterica* NBRC13245 | LB / 37°C / 17 h with shaking | SCDLP agar / 37°C / 48 h |
| *Staph. aureus* NBRC12732 | LB / 37°C / 17 h with shaking | SCDLP agar / 37°C / 48 h |
| *B. cereus* NBRC15305 | LB / 37°C / 17 h with shaking | SCDLP agar / 37°C / 48 h |
| *B. subtilis* ATCC6633 | LB / 37°C / 17 h with shaking | SCDLP agar / 37°C / 48 h |
| *K. rhizophila* NBRC12708 | LB / 37°C / 17 h with shaking | SCDLP agar / 37°C / 48 h |
| *A. acidoterrestris* NBRC108913 | Specific broth (No. 323)** / 45°C / 17 h with shaking | No. 323 agar / 45°C / 48 h |
| *Sporolact. inulinus* NBRC13595 | MRS broth*** / 37°C / 24 h in an anaerobic chamber (no shaking) | MRS broth / 37°C / 72 h in an anaerobic chamber |
| *S. cerevisiae* NBRC1136 | Sabouraud broth**/ 28°C / 24 h with shaking | Sabouraud agar††† / 28°C / 72 h |
| *C. cladosporioides* IFM63149 | PDA†/ 25°C / 12 days | PDA / 25°C / 72 h |
| *B. fulva* NBRC31767 | PDA / 25°C / 12 days | PDA / 25°C / 72 h |
| *E. lapidosum* NBRC6100 | PDA / 25°C / 12 days | PDA / 25°C / 72 h |

*Luria-Bertani medium (Lennox; Nacalai Tesque Inc., Kyoto, Japan); **See Materials and Methods; ***MRS broth for microbiology (Sigma-Aldrich Co., LLC, St. Louis, MO, USA); †Potato dextrose agar (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan); ††Nihon Pharmaceutical Co., Ltd. Tokyo, Japan; †††Nissui Pharmaceutical Co. Ltd.

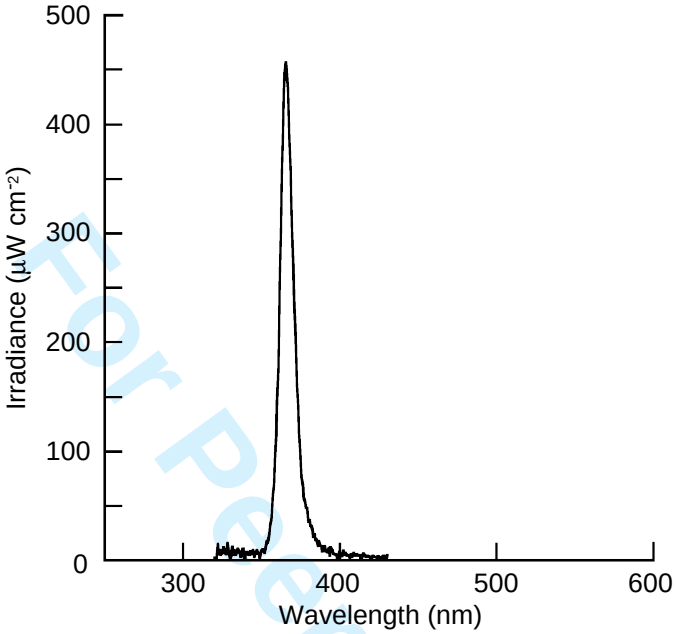


**Fig S1** Emission spectrum of the UV-A LED as used in this study; the spectrum exhibited a maximum at 365 nm, which was measured at a distance of 30 mm between the illumination source and the UV meter.

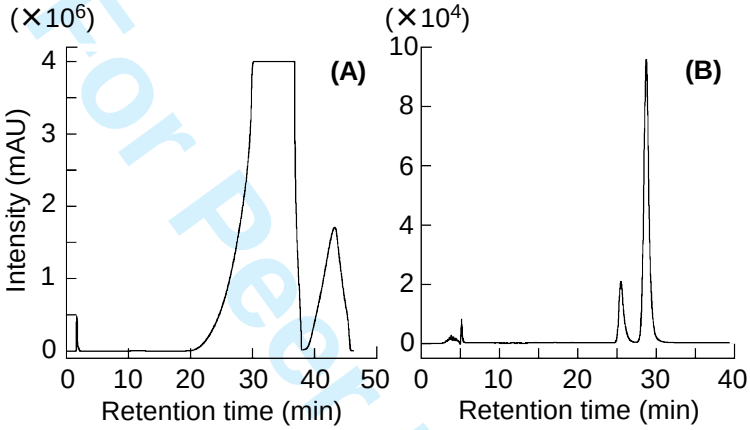


**Fig S2** HPLC chromatogram of FA prepared by UV-A exposure: before separation (A), and after separation and purification of the *cis*-form (B).

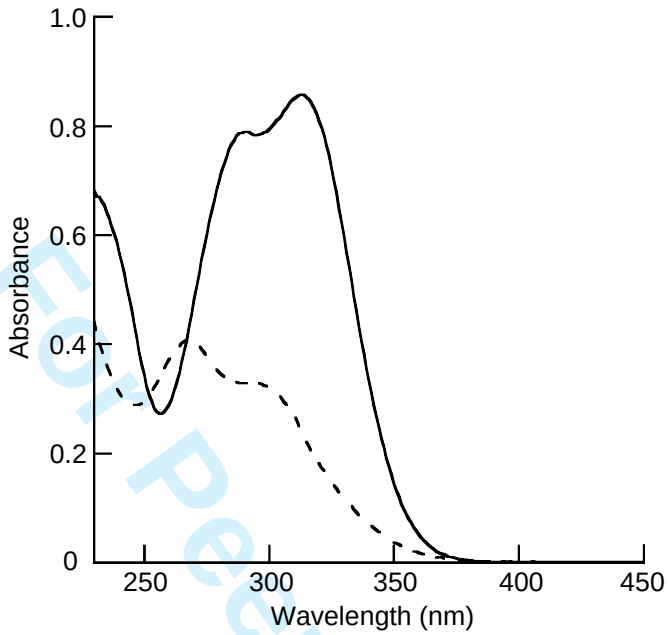


**Fig S3** UV-visible absorption spectra of FA (*trans*-form, solid line) and isomerized FA (mixture of 93% *cis*-form and 7% *trans*-form, dashed line). Samples were dissolved at 50 μM in 0.4% DMSO.

**Authors' answers against Reviewers' comments**

I indicated all correction points (changes and edits) by using the highlighter tool to highlight the changes in yellow in our manuscript.

**REFEREE COMMENTS TO AUTHORS (Reviewer 1)**

Without underestimating the author's effort, but taking their experience form previous cited scientific reports, this study would have been more completed if trials of food liquids would had been performed. In that way, it will give us a clue on the effect of the organic content of these foods items (juices) or if ferulic acid would have affected the quality attributes of the juices.

**Authors' answer**

Thank you for giving me some advices related to next challenge. In this report, the inactivation experiments were carried out in sterile water of high UV transmittance. In future work, we will show the potential use of the combination of UV-A light and FA in complex food surfaces.

**Comment of reviewer 1 (in general)**

Page 2, line 21. Use the word "microorganisms" instead of "microbes".

**Ans.** I corrected the term of "microbes" to "microorganisms" in our manuscript, report title, and Tables 1 and S1 (Lines 1, 63, 96, 98, 208 and 359).

**Comment of reviewer 1 (in general)**

Page 2, line 20. I prefer to read the impact of food/juice industry when using this proposed method of disinfection.

**Ans.** I rewrote "SIGNIFICANCE AND IMPACT OF THE STUDY" (Line 21-28).

**Comment of reviewer 1 (in general)**

Page 2, line 29. The term "sterilization" is not the right one. I suggest "decontamination" and/or "disinfection".

**Ans.** I corrected the term of “sterilization” to “disinfection” in our manuscript (Lines 30, 47).

**Comment of reviewer 1 (in general)**

Page 2, line 31. The word "killing" is a general term, I would prefer a more "biological" term: "reducing" and/or "eliminating".

**Ans.** I corrected the term of “killing” (Lines 32, 37, 38, 145, 181, 186, 187 and 200).

**Comment of reviewer 1 (in general)**

Page 2, line 40. Delete the word "minimum".

**Ans.** I deleted “minimum” (Line 41).

**Comment of reviewer 1 (in general)**

Page 19. Table 1. Do not abbreviate scientific names.

**Ans.** I indicated scientific names for microorganisms in Table 1 (Page 19).

**REFEREE COMMENTS TO AUTHORS (Reviewer 2)**

Major concerns.

In my opinion, the section corresponding to significance and impact of the study is not written according to the instructions given in the author guidelines of the journal; it has been written more as a kind of abstract. Please, check the instructions and modify.

**Authors’ answer**

I rewrote “SIGNIFICANCE AND IMPACT OF THE STUDY” (Line 21-28).

**Comment of reviewer 2**

Lines 117-118. According to your citation of Graf (1992), is not possible that trans ferulic acid had been isomerized to cis-form by your UV treatment, therefore, you had actually had cis-form in both cases?

**Authors’ answer**

Please see supporting information (Text S1, Page 23-25).

*cis*-FA was generated by isomerization using UV-A exposure of *trans*-FA, and then the *cis*-form was separated from the parent isomer using HPLC. We confirmed that the separated FA was composed of 93% *cis*-form and 7% *trans*-form on the basis of the HPLC analysis, as demonstrated by detection of the α-H of double bonds in each propenoic acid moiety corresponding to *cis*- and *trans*-FA; the coupling constants were 13.0 Hz (δ, 5.73 ppm; doublet peak) and 15.9 Hz (δ, 6.36 ppm; doublet peak), respectively.

**Comment of reviewer 2**

Line 155. On complete inactivation. How are authors sure that complete inactivation was achieved without performing enrichment tests that can show that no survivors existed?

**Ans.** I thought that the word of "complete" was not appropriate, because the inactivation means that viable cell count was below 10 CFU $ml^{-1}$ (the lower limit of detection). In line 153, I corrected the term of "Complete inactivation" to "Potent inactivation".

**Comment of reviewer 2 (Minor concerns)**

Line 40. Delete "Minimum".

**Ans.** I deleted "minimum" (line 41).

Line 48. More versatile than what?

**Ans.** I corrected "a more versatile UV source" to "a versatile UV source" (Line 48).

Line 58. Delete "D."

**Ans.** I deleted "D" (Line 58)..

Lines 59-61. Which is the advantage of UV-A over UV-C in this regard?

**Ans.** UV-A is superior to UV-C in this regard. A previous report has described no differences of Vitamin C content in cabbage tissue before and after UV-A irradiation (Aihara M., et al., J Med Invest (2014) 61, 285-290).

Lines 67, 122. Avoid using very personal terms, write in a more impersonal fashion.

**Ans.** I wrote in sentences as follows (Lines 67-70):

To increase the inactivation efficiency of UV-A, the synergistic bactericidal activity of the

combination of UV-A light and organic substrates, a quaternary ammonium salt (Shirai et al. 2014), and natural compounds [coumaric acid and ferulic acid (FA)] and their derivatives, has been investigated in our laboratory (Shirai et al. 2015a, 2015b).

**Ans.** I wrote in sentences as follows (Line 121):

Photoantimicrobial assays were performed at FA concentrations below its MIC.

Line 78. Delete "D" before "Paiva".

**Ans.** I deleted "D" (Line 76).

Lines 80-93. Write in few lines the goal of this research at the end of the introduction in order for the reader to catch it quickly and clearly, avoiding mixing them with the justification of testing the microorganisms used in the research, which can be placed before the goals.

**Ans.** Please see lines 80-92 in manuscript.

Lines 132 and elsewhere. It is not necessary to repeat each time data on concentration, fluences, etc. It is enough to do it at first mentioning.

**Ans.** I rewrote sentences as follows (Lines 130-131):

the combination of FA with UV-A yielded significant ($P < 0.01$) decreases in the viable cell count.

Line 139 and elsewhere. Use a single letter for generic names.

**Ans.** The abbreviation for those generic names, *Salm., Staph., and Sporolact.*, complies with "common generic names" indicated in Author Guidelines for this journal (Lines 137 and elsewhere).

Line 183. Use only two decimals.

**Ans.** I corrected 0.007 to 0.01(Line 181).

Line 198. Separate conclusions in a single paragraph for better exposure to the reader.

**Ans.** I separated the conclusions in a single paragraph (Line 197).

Line 212. Microbial strains must be included in this section. You could cite table 1 to this end.

**Ans.** I wrote in sentence as follows:

Those strains are listed in Table 1 (Line 210).

**REFEREE COMMENTS TO AUTHORS (Reviewer 3)**

This manuscript is useful and helpful to develop new types of antimicrobial reagents, especially combination with UV-A without hazardous UV-C is interesting. On the other hand, why does UV-A enhance the antimicrobial activity of FA? This question is a major issue to the next research. I hope you experimentally solve this question soon.

**Ans.** I am going to solve the synergistic bactericidal mechanism in a next report.