Table 1. MICs on the first, fifth, and tenth exposure of each bacterial species to antibacterial agents.

Staphylococcus aureus				**Enterococcus faecalis**			
Drug	**MIC (μg/mL)**			**Drug**	**MIC (μg/mL)**		
	Initial	**5th**	**10th**		**Initial**	**5th**	**10th**
AMX*	0.12	1	2	**AMX***	0.5	1	2
CFPN*	0.5	128	128	**CFPN***	8	128	128
EM*	0.5	4	8	**EM***	2	4	8
OFLX*	0.5	4	4	**OFLX**	2	4	4
CLDM*	0.25	16	32	**CLDM**	16	16	32
CPFX*	0.5	2	2	**CPFX**	1	2	2
MINO*	0.25	2	8	**MINO***	2	8	16
Escherichia coli				**Streptococcus salivarius**			
Drug	**MIC (μg/mL)**			**Drug**	**MIC (μg/mL)**		
	Initial	**5th**	**10th**		**Initial**	**5th**	**10th**
AMX	4	8	8	**AMX***	0.03	0.03	0.12
CFPN	1	2	2	**CFPN**	<0.015	<0.015	<0.015
EM	32	64	64	**EM**	0.06	0.06	0.12
OFLX*	0.06	0.12	0.48	**OFLX**	2	4	4
CLDM	128	128	128	**CLDM**	0.06	<0.015	<0.015
CPFX	0.015	<0.015	<0.015	**CPFX**	1	2	2
MINO*	0.5	4	16	**MINO**	<0.12	<0.12	<0.12

Each value represents the mean of duplicate determinations.
Asterisks indicate induction of bacterial resistance to corresponding antibacterial agents as defined by an increase of four times or more in MIC over the initial MIC.
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above, an approximate 2-log reduction in viable counts was observed at the first exposure of each bacterial species to treatment with photolysis of H_2O_2. Of the three bacterial species, *P. aeruginosa* and *A. actinomycetemcomitans* showed a relatively high susceptibility to this treatment because a laser light irradiation time as short as 10 s for *P. aeruginosa* and 30 s for *A. actinomycetemcomitans*, was sufficient for achieving a 2-log reduction in viable counts. Repeated exposure of these two bacterial species to treatment with photolysis of H_2O_2 resulted in a relatively large fluctuation in the antibacterial effect compared with *S. mutans* and the four bacterial species (*S. aureus*, *E. faecalis*, *E. coli*, and *S. salivarius*) shown in Figure 3. However, even in the two species *P. aeruginosa* and *A. actinomycetemcomitans*, no development of bacterial resistance to treatment of photolysis of H_2O_2 was observed during 40 times of exposure. For *S. mutans*, as was the case with the former four bacterial species (*S. aureus*, *E. faecalis*, *E. coli*, and *S. salivarius*), the magnitude of reduction in viable counts hardly deviated from the range of 2- to 3-log order during repeated treatment up to 40 times.

Quantification of hydroxyl radicals generated by photolysis of H_2O_2

Laser irradiation of H_2O_2 generated an ESR signal of DMPO-OH. The presence of the spin adduct was confirmed by hyper fine coupling constants of $a_N = a_H = 1.49$ mT for DMPO-OH [17]. The yield of DMPO-OH increased linearly with the laser irradiation time, and the generation rates of DMPO-OH (slope values of lines) also increased with the concentration of H_2O_2 (Fig. 5). When H_2O_2 at concentrations of 250, 500, and 1000 mM was irradiated with the laser light for 30 s, the yields of DMPO-OH were 12.8, 22.5, and 41.6 mM, respectively.

Discussion

The present study showed that repeated exposure of bacteria to disinfection treatment with photolysis of H_2O_2 did not induce bacterial resistance to this treatment. With regard to the antibacterial agents tested, in all of the agents tested, at least one of the four bacterial species resistant to the agents was observed with repeated exposure. As mentioned above, monitoring MICs of the agents after serial passage of the culture through subinhibitory concentrations of the agents has proven effective for assessing the risk of developing bacterial resistance [11–13]. Bacteria were cultivated under drug-free conditions prior to each susceptibility assay in the present study (Fig. 1a). The setup of the assay protocol was designed in this manner to be in accordance with susceptibility testing for disinfection treatment with photolysis of H_2O_2. Because continuous or serial exposure of bacteria to treatment with photolysis of H_2O_2 would cause a lethal effect, the serial passage technique could not be applied. Therefore, bacteria were cultivated prior to each susceptibility assay under partially bactericidal conditions, which were obtained by adjusting the laser light irradiation time (Fig. 1b). Even when cultivation was performed in advance between each susceptibility assay, repeated exposure of bacteria to subinhibitory concentrations of antibacterial agents resulted in development of bacteria that were resistant to the agents. Of the four bacterial species tested, increases in MICs were more prominent in *S. aureus* and *E. faecalis* than in *E. coli* and *S. salivarius*. The reason for the difference in the magnitude of drug-resistance induction among bacterial species cannot be explained at the present time. In addition, only one strain for each bacterial species was tested. Therefore, the conclusion that this difference was species dependent cannot be made. Nonetheless, to

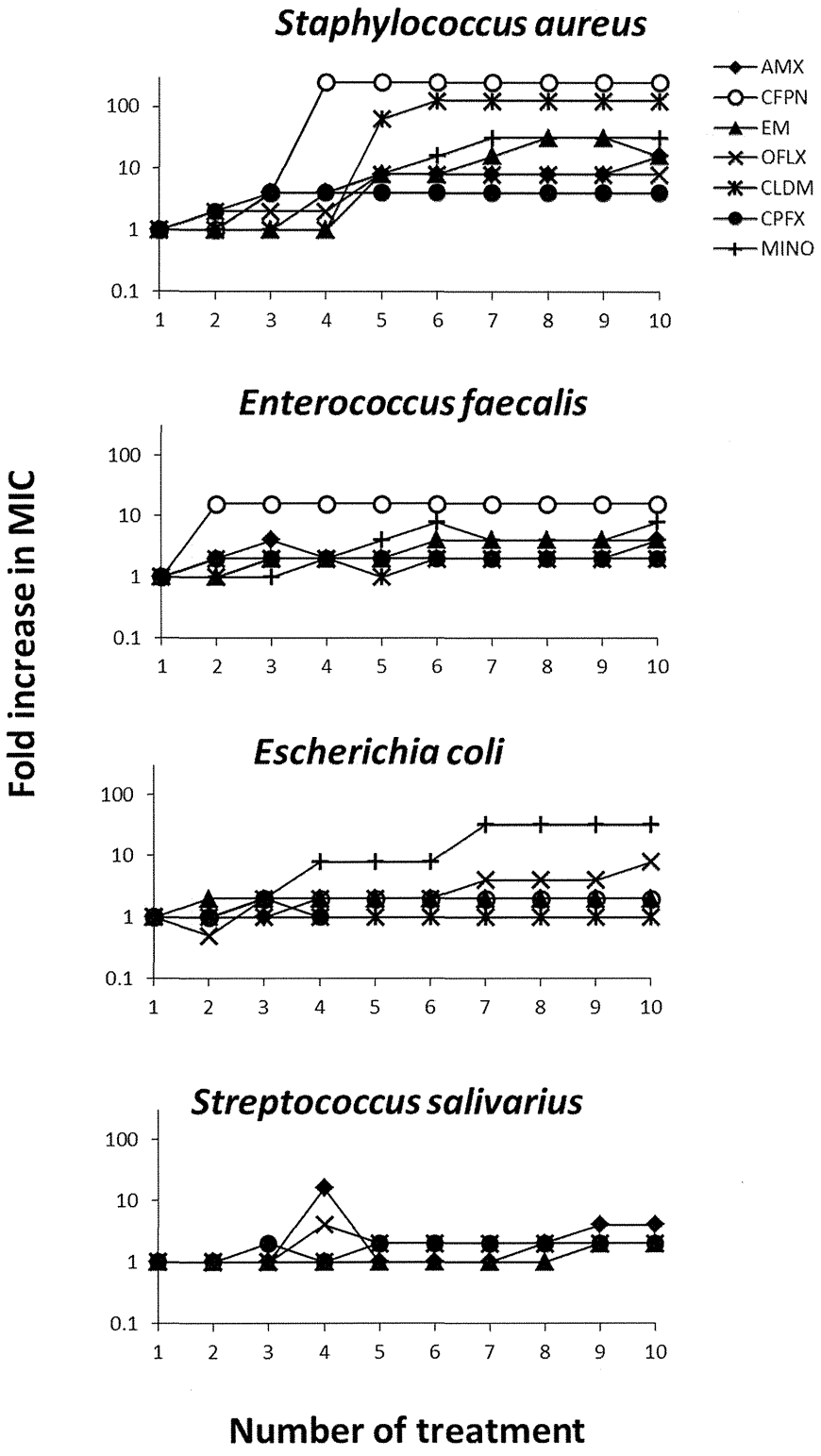

Figure 2. Fold increases in MICs of antibacterial agents against four bacterial species during exposure to these agents. Each bacterial species was exposed 10 times. Each initial MIC is regarded as 1 MIC. Each value represents the mean of duplicate determinations.
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a greater or lesser extent, any of the bacterial species tested became resistant to one or more antibacterial agents tested. Under a similar assay protocol, disinfection treatment with photolysis of H_2O_2 did not result in development of resistance to this treatment in any of the four bacterial species, even after 40 exposures. With regard to the other three bacterial species, *P. aeruginosa*, *S. mutans*, and *A. actinomycetemcomitans*, disinfection treatment with photolysis of H_2O_2 also did not lead to development of resistance.

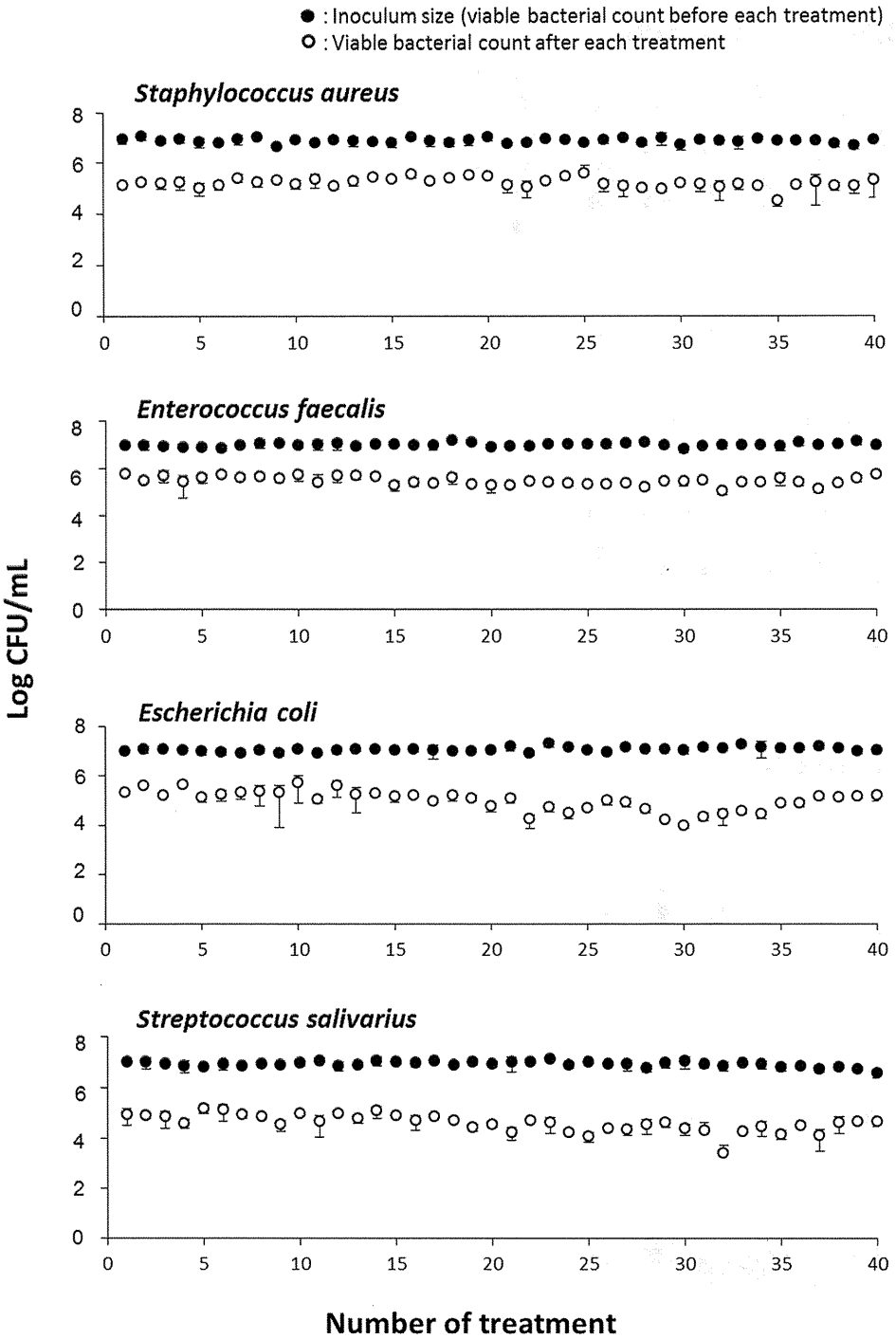

Figure 3. Changes in the antibacterial effect of disinfection treatment with photolysis of H_2O_2 in four bacteria. *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Streptococcus salivarius* were exposed 40 times to disinfection treatment. Each value represents the mean ± standard deviation (n = 3).
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Susceptibility of *P. aeruginosa* and *A. actinomycetemcomitans* to repeated treatment of photolysis of H_2O_2 fluctuated compared with the other bacterial species. In the case of *P. aeruginosa*, this was possibly due to a higher sensitivity of this bacterium than that of the other bacterial species. This could be because a laser light irradiation time as short as 10 s was sufficient to achieve a 2-log reduction in viable counts. With regard to *A. actinomycetemcomitans*, one of the possibilities for causing fluctuation might be that the bacterium was cultured under anaerobic conditions following exposure to oxidative stress by hydroxyl radicals, as well as its relatively high sensitivity to disinfection treatment. However, since such fluctuation was not observed in *S. mutans* which was also cultured under anaerobic conditions, effect of anaerobic culture conditions might not be so important.

In general, bacterial resistance is mediated through inactivation of drugs, mutation of active sites of drugs, and/or inhibition of

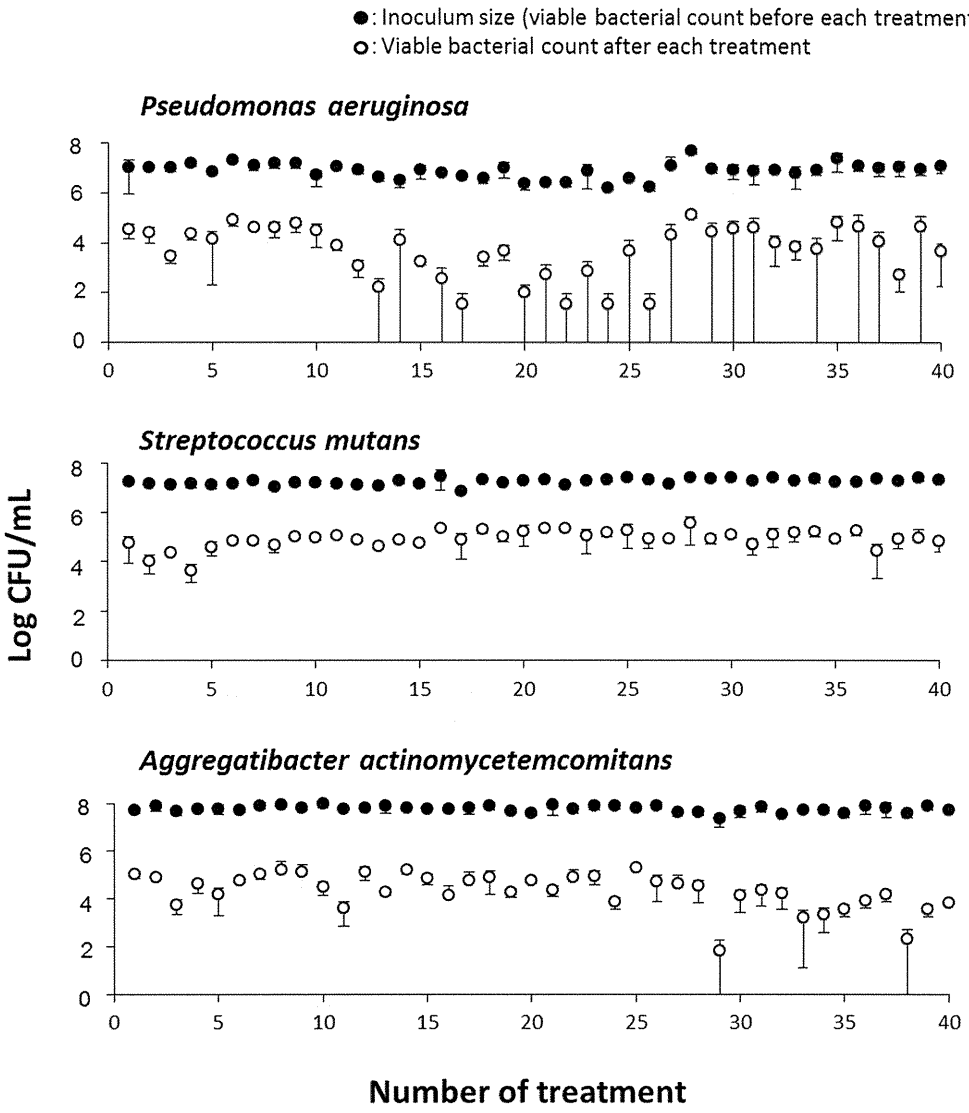

Figure 4. Changes in the antibacterial effect of disinfection treatment with photolysis of H_2O_2 in three bacteria. *Pseudomonas aeruginosa, Streptococcus mutans,* and *Aggregatibacter actinomycetemcomitans* were exposed 40 times to disinfection treatment. Each value represents the mean ± standard deviation (n = 3).
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drug-accession to active sites. In addition, bacteria resistant to more than two classes of antibiotics, which are categorized as multidrug resistant, have become a serious problem in the hospital environment. Multidrug resistance may be mediated by extra-chromosomal genetic elements or by overexpression of resistance genes in response to selective pressure [18]. In contrast to susceptibility testing for antimicrobial agents, repeated exposure of the seven bacterial species to disinfection treatment with photolysis of H_2O_2 did not decrease bacterial susceptibility to this treatment. This finding suggests that the risk of inducing bacterial resistance by disinfection treatment is low. In the case of photodynamic antimicrobial chemotherapy (PACT) in which exposure of a photosensitizer to light results in the formation of oxygen species (e.g., singlet oxygen and free radicals), causing microbial cell death, the development of resistance to photodynamic antimicrobial chemotherapy appears to be unlikely. This situation occurs because, in microbial cells, singlet oxygen and free radicals interact with several cell structures and different metabolic pathways [7]. The active ingredient of the disinfection treatment in the present study was the hydroxyl radical, which was laser irradiation time-dependently generated by photolysis of H_2O_2, but not H_2O_2, because exposure of bacteria to 3% (w/v) H_2O_2 without laser irradiation for up to 120 s did not show any bactericidal effect. In studies on PACT, Guiliani et al. studied the possible development of bacterial resistance to PACT after 20 treatments in three major human pathogens, *P. aeruginosa*, *S. aureus*, and *Candida albicans* [10]. All samples were illuminated with a fluence rate of 50 mW/cm^2 for 10 min, and the condition allowed the pathogens survive the PACT. They demonstrated that 20 consecutive PACT treatments did not result in any resistant mutants. Similarly, Tavares et al. demonstrated that the bacteria did not develop resistance to the photodynamic process [9]. In their study, *Vibrio fischerithe* and *E. coli* were subjected to 10 repeated PACT. In their PACT with white light irradiation at 40 W/m^2 for 25 min, 1 log unit of surviving bacteria was achieved. In our study, the disinfection treatment with photolysis of H_2O_2 was carried out on the second time scale. Since we have developed the disinfection treatment with photolysis of H_2O_2 to achieve highly effective bactericidal activity, the

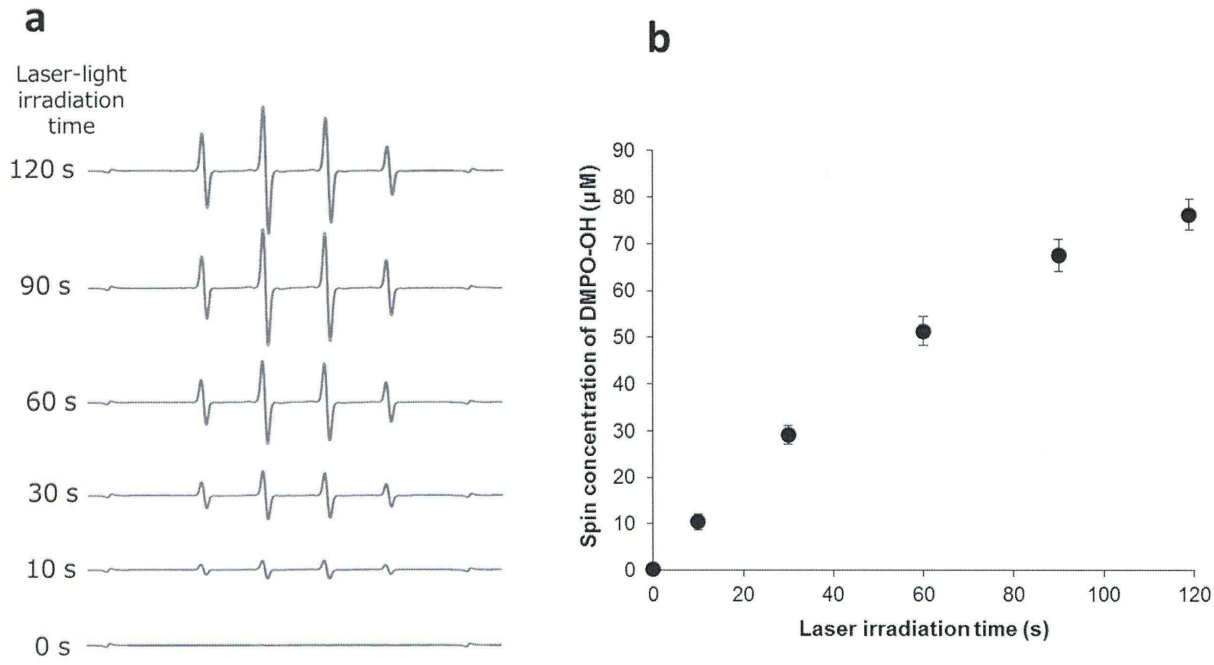

Figure 5. Representative ESR spectra and the yield of DMPO-OH obtained by laser-light irradiation of 3% H_2O_2. (a) ESR spectra and (b) DMPO-OH yields are shown. Each value in (b) represents the mean ± standard deviation (n = 3).
doi:10.1371/journal.pone.0081316.g005

disinfection treatment applied in the present study has an ability to kill pathogenic bacteria including *S. aureus* and *E. faecalis* with a >5-log reduction of viable counts within 3 min [1], indicating that it is difficult to get bacteria surviving the disinfection treatment after 3 min treatment. To evaluate the risk of inducing bacterial resistance, surviving bacteria is needed to be subcultured for the next passage. That is a reason for that the treatment was carried out on the second time scale up to 120 s. To study the risk of developing bacterial resistance in this manner, not only the disinfection treatment with photolysis of H_2O_2 but also PACT was set to exert sublethal effect by controlling the treatment time although the disinfection treatment in the present study was carried out on the second time scale and PACT on the minute time scale. Thus despite the somewhat difference in the treatment time between the two, it is assumed that the experimental conditions were comparable each other. Therefore, as is the case with PACT, it was expected that no bacterial resistance was induced by hydroxyl radicals. Anti-oxidant enzymes, such as superoxide dismutase and catalase, protect against some reactive oxygen species, but not against hydroxyl radicals. There is the possibility that catalase in bacterial cells may affect H_2O_2, resulting in a reduced amount of hydroxyl radicals. However, this would be negligible because 3% H_2O_2 is a high enough concentration that bacteria should not be degraded by their inherent catalase. To further confirm the low risk of developing bacterial resistance, more bacterial strains including drug resistant mutants should be evaluated since only one strain of each bacterial species was tested in the present study.

Considering the emergence of antibiotic-resistant strains in recent years, disinfection treatment with photolysis of H_2O_2 appears to be a potential alternative for existing antimicrobial agents because of its low risk of inducing bacterial resistance.

Author Contributions

Conceived and designed the experiments: YN TK KN KS. Performed the experiments: HI YO KN MS. Analyzed the data: YN HI KN. Contributed reagents/materials/analysis tools: HI YO KN MS. Wrote the paper: YN.

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