Evaluation of gaseous ozone virucidal efficacy against virus contamination on foods and packaging materials

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Abstract

Diffusing of virus can occur through indirectly by contacting with food and surfaces of packaging materials being in the infected environment. Ozone proved effective in decontaminating pathogenic microorganisms on foods. To this end, this study explored the virucidal efficacy of gaseous ozone for foods and packaging materials by using three widely accepted viral surrogates (φ6, MS2, and φX174). The φ6 was highly disinfected (≥1.6-log reduction and 90% inactivation) by gaseous ozone (200 mg/h, 3 min) on foods except for peanut kernel. As observed, the same treatment had a little effect (≤ 0.19-log and 50% inactivation) on φX174 and MS2 on peanut kernel, even at the longer durations under the tested conditions (200 mg/h, 5 min, 6 min). On average, the adsorbed virus surrogates on soybean were better disinfected (≥70% inactivation). Hence, gaseous ozone could be a reliable way for inactivating φ6, φX174, and MS2 on soybean; however, continued efforts are needed to make gaseous ozone more applicable to peanut kernel. Gaseous ozone was effective for the inactivation of φ6, φX174, and MS2 on packaging materials. These results were encouraging as ozone treatment significantly reduced φ6, MS2 (≥1.19-log and 90% inactivation) on kraft paper, plastic, and glass panel, in which φX174 was disinfected over 70%. To conclude, gaseous ozone was an important technology for surface detoxification of foods and packaging materials because it could effectively control φ6, MS2 and φX174, but reducing the levels of viral surrogates on peanut kernel required new conditions.

Introduction

Globally, a survey conducted by Havelaar et al.[1] has shown that food-origin disease caused by infectious agents in 2010 including 420,000 deaths and 600 million cases of disease. Viruses, identified as one of the most reported causes of disease outbreaks worldwide, were mainly associated with shellfish, water, berries, shallots, fresh or frozen meat.[2] In an investigation into Norovirus (NOV), Velebit et al.[3] found it and acute gastroenteritis to be closely linked. Hepatitis A virus (HAV) were also involved in the majority of food-related epidemic connected with agricultural products between 2009 and 2015, including a large-scale outbreak of HAV caused by semi-dried tomatoes in several Australian states in 2009–2010 and a symptomatic outbreak of HAV linked to consumption of pomegranate arils in the United States.[4,5] Recently, the most troublesome epidemic viruses causing serious human diseases and closely related to food production, preparation, and food packaging are coronaviruses. From December 2019 to August 30 2021, already, as the SARS-CoV-2 virus infected well over 220 million people worldwide, and its caused disease (COVID-19) has statistically resulted in a death toll exceeding 4.5 million.[6] A serious problem could be noticed by a recent research by Chin et al.[7] showed that SARS-CoV-2 survival can be maintained on a plastic surface with a relative humidity of 65% at room temperature, and completely inactivated on stainless steel surfaces following 7 days survival. Therefore, there is a need for low-cost and efficient sterilization methods from which the disinfection of foods and packaging materials are positively boosted.

Model phages were already developed and applied to a variety of viral substitutes because they are easier to produce in high volumes using little funding and are safe for laboratory personnel, requiring fewer biocontainment precautions.[8,9,10] Importantly, a wide range of phages with distinct characteristics (e.g., enveloped and non-enveloped, dsRNA and ssRNA, dsDNA and ssDNA) are used to characterize a broader spectrum of eukaryotic viruses and their resistance to foodborne disinfectants. Upon repeated pre-experiments, three model phages were finally taken: MS2, φX174 and φ6. MS2 is an accepted model of NOV which is now considered the international standard for evaluating water quality for waterborne transmission of human enteroviruses.[11,12] φX174 was chosen on account of its genetic material (ss DNA) and how easy it is to use.[13] Finally, owing to lipid envelope, φ6 is considered an excellent surrogate for influenza, such as Influenza A virus, H7N1 and Venezuelan equine encephalitis virus.[14]

Microbial interactions, infrared techniques and cold plasma as emerging non-thermal disinfection technologies have been extensively studied.[15] Although such methods have numerous commercial benefits in an industrial context, they are typically not applied on a broad scale given the high cost and maintenance of equipment. Ozone is a sanitization technology brimming as a viable alternative to chloric-compound, H_2O_2, and surfactant. As an effective disinfectant, inactivating viruses, bacteria, fungi, and protozoon, besides, it breaks down quickly and leaving no residues on foods or packaging materials.[16] Detailed study showed that ozone provided effective control of Norwalk virus found in drinking water, with ozone at pH 7 (0.37 ppm) for 5 minutes at 5°C achieving an effective reduction in concentration of more than 3-log in 10 seconds.[17] It was recognized as a non-pharmaceutical treatment against coronaviruses and other deadly viruses in the human body and the environment.[18] All of the studies reviewed here support the idea that ozone could there potentially be one of the safest biocides employed in the food industry. Hence, this study was designed to evaluate the effectiveness of gaseous ozone in disinfecting virus surrogates on foods and packaging materials.

Materials And Methods

Preparation of model phages and host bacteria

All host strains were provided by ATCC (American Type Culture Collection) and NBRC (NITE Biological Resource Centre). Their characteristics and growing conditions are listed in Table 1. Just before the experiment started, each strain was grown twice consecutively in tryptic soy broth (TSB), activated at 26 °C or 37℃ for 24 hours of incubation. On finishing the incubation period, bacteriophages were spread germ-free on sterile soy agar (TSA) each, and incubated in an aerobic manner at 26 °C or 37°C for 24 hours. Grown colonies were subsequently selected and re-cultured as described previously from a single plate of each strain. Once incubated, the slants were saved at 4 –5 °C and served as a working slant for the entire project.

DNA extraction, amplification and 16S-rRNA sequencing

The host bacteria were grown overnight in a liquid Nutrient Broth at 26°C *(P.svringe* NBRC14084) or 37°C *(E.coli* ATCC13706 and *E.coli* ATCC15597). The CTAB method was used for the isolation of genomic DNA as described by Akinyemi and Oyelakin.[19] The 16S-rRNA gene was amplified using template DNA extracted from genomic DNA. The PCR mix comprised of 5 µL of 10 × buffer, 1 µL of 2.5 mMdNTPs, 2 µL 5 mM forward and reverse primers, 1 µL of 5 units/µL Taq containing 1 µL of template DNA and 40 µL of distilled water to make-up 50 µL reaction mix. Amplification of the nearly complete 16S-rRNA gene with universal 27f (5'-AGAGTTTGCCTGGCTCAG-3') and 1492r (5'-TACGGCTACCTTGTTACGACTT-3') primers was carried out by PCR in 30 cycles denaturation (94℃, 60 s), annealing (55℃, 60 s), and elongation (72℃, 30 s), and a final polymerization step (72℃, 10 min). Detected by 2% agarose gel electrophoresis, the PCR products were purified using a Direct PCR purification kit in accordance with the manufacturer's guidelines (B639289, Shanghai, China). The obtained final products were sent to Qingke Biotechnology Co., Ltd (Beijing, China) for sequencing.

Effect of gaseous ozone to viral substitutes in water, gas phase

A sketch of the apparatus used in this research are presented as Fig. 1. An approximately 1.5-liter stainless steel chamber (15 cm × 10 cm × 10 cm) was installed as a sterilization box. On one hand the ozone gas flowmeter (WT-RSL-YT, Nanjing China) was connected with the ozone generator (ARDM0.3 Shanghai China), on the other hand it was connected to the top of the box through a polypropylene-random pipe and extended to bottom. To control for bias caused by air, the sterilization box was equipped with a vacuum air pump on the side.

Stainless steel sheets were autoclaved and put in 5 mL of each virus stock solution. Three model phages finally held the following titer: 7.21 log units, 6.68 log units and 7.51 log units for φ6, φX174 and MS2, respectively. To determine ozone gave the effect on bacteriophage in gas phase, the following procedures were used. The contaminated stainless steel sheets were placed horizontally in the center of the disinfection box. After the multifunctional generator started, the transfer switch was not turned on until 200 mg/h of gaseous ozone produced steadily, avoiding excessive or insufficient amount of gas to increase experiment bias. Finally, the samples were exposed to 200 mg/h gaseous ozone for 0, 1, 2, 3, 4, 5 or 6 min. To assess whether and how resistance of bacteriophages was affected and changed in water phase, we exposed the samples to water filled with ozone and injected stable gaseous ozone for 0, 1, 2, 3, 4, 5 or 6 min. Quantified the effect of gaseous ozone to viral substitutes by log reduction and inactivation following varied time treatment with constant concentrations of gaseous ozone.

Inoculation of samples

A batch of foods and packaging materials (Table 2) were measured and placed in a taker-bag (50 mm × 100 mm). For inoculation, 5 mL of the viral stock solution was added to those bags containing foods and packaging materials. Samples and inoculum were mixed with a slow shaking of the bag for 30 minutes allowing the sample to be uniformly infected while preventing the bag from breaking. Notably, foods were each inoculated superficially, avoiding scarred foods as this is a known way to harbor and internalize

pathogens.[20] Rather than concentrating on inactivating the bacteria inside the product, we focused only as much on the bacteria on the surface of the product in this study. Samples were loaded with target bacteria at a concentration of 4-6 log units. Once infected, samples were picked up from the bags and counted for bacteria which verified the initial concentration of inoculated pathogens. Subsequently, the infected samples were dried in a cabinet at 22°C for 2 h before ozone gas treatment. The samples were dried, and left at 4°C overnight ensuring the virus particles were dry and adhered.

Treatment of samples with gaseous ozone

The results in previous chapter established theeffect of gaseous ozone to viral substitutes in water, gas phase. In this chapter, therefore, moves on to discuss the effect on samples with ozone at 3,5,6 min for infected samples by φ6, φX174 and MS2, respectively. When a gas flow with 200 mg/h of ozone was passed through the gas flowmeter and detected. The samples were quickly pushed into the box laterally and then disinfected for 3, 5 and 6 min based on our previous tests, respectively. After disinfection, not only did the excessive ozone in the box required released from the bottom, it also needed a complete cleaning and disinfection program. Only in this way can we better avoid bias caused by interactions between samples.

Statistical analysis

The experimental data were transformed to log values, then log reduction values were expressed for each sample group, by using the initial log value of each pathogen minus the processed log value. For inactivation efficacy, the raw data were collated and presented as a decreased percentage of virus titer as accounted by the formula:

$$Inactivation(\%) = 1 - \frac{Virus\ titer\ on\ tested\ products\ after\ treatment}{Initial\ virus\ titer\ on\ tested\ products} \times 100$$

Data management and analysis were performed using SPSS v.20 (SPSS, Chicago, IL, USA), Between-mean differences were calculated using Duncan's multiple range tests. Results with P values of < 0.05 were considered significant and indicated with different letters. Each experiment was repeated five times independently removing the maximum and minimum values.

Results

Bacterial PCR amplification results and sequence analysis

After incubating the stored strain at 37ºC or 26ºC for 24 hours, PCR of purified host bacterial DNA with universal primers showed a 1500 bp band on the gel that matched the expected length of the amplified fragment. To compare the amplified 16S-rRNA gene sequences, using the National Center for Biotechnology Information (NCBI) gene bank which lied in the Basic Local Alignment Search Tool (BLAST). The result of BLAST showed that *P. svringe* NBRC 14084 was 98% similar to *Pseudomonas*

congelans strain P538/23. *E. coli* ATCC15597 and *E. coli* ATCC13706 were 99% similar to *Escherichia fergusonii* ATCC 35469. The contamination relating to bacteria strain was common and was easily affected by deviations in storage and culture conditions. In this study, the rejuvenated strains were therefore biochemically identified, and the test proved that they were not contaminated by miscellaneous bacteria and were well preserved.

Effect of gaseous ozone to viral substitutes in water, gas phase

We evaluated the effect of ozone in gas and water phase to inactivate the φ6, φX174 and MS2. In order to assess the effect, the experimental data were presented through log reduction and inactivation. The disinfected effect of ozone in water phase increased as the treatment time progressed. In gas phase, we observed a similar trend. Ozone in gas and water phase reduced the titers of φ6 by 1.08 and 0.88-log after a 1 min treatment, respectively (Fig 3). Increasing the exposure time to 2 min, the water and gas phase containing ozone achieved 3.30 and 1.40-log on φ6, respectively. Improving the exposure time to 3 min caused a significant increase in log reduction (4.01 and 7.21-log) for φ6, but a significant difference in inactivation (99.96% and 100%) between gas and water phase was not found. Notably, under prolonged treatment, φX174 and MS2 were reduced in a similar trend to φ6 in water and gas phase (Fig 3 and 4), where both pseudoviruses were destroyed (reduction 4-log) after 5 and 6 min, respectively.

Impact of gaseous ozone to viral substitutes on food surface

The reduction of above viral substitutes (φ6, φX174 and MS2) on five different food matrices: dried wolfberry, fresh wolfberry, grape, peanut kernel and soybean was tested. The results of φ6 log reduction values on samples are shown in Fig.5, and the inactivation can also be observed. Ozone used to disinfect soybeans with the concentration of 200 mg/h achieved a maximum reduction of 2.07-log and inactivation of 99.11% on φ6 after a 3 min contact time. From this data, we can see that peanut kernels resulted in the lowest value of reduction (0.55-log) and inactivation (71.86%). Obviously, the reduction of φ6 on the surface of other foods reached more than 1.6-log and the inactivation was over 97%. Similarly, only trace amounts of reduction (0.19-log) and inactivation (50%) of φX174 were found on peanut kernel surface compared with other food matrices (Fig.6). When samples were treated with a constant concentration of ozone, the reduction of φX174 was reduced on dried and fresh wolfberries by 0.65-log and 0.85-log, and by 0.85-log, 0.86-log on grapes and soybeans, respectively. From the Fig.7, it can be seen that the reduction (0.16-log) and inactivation (30.41%) of MS2 on peanut kernel surface was by far the lowest. What is apparent about the data in this figure is that the reduction of MS2 was reached by 1.16-log and 0.71-log on soybeans and fresh wolfberries after a 6 min contact time, respectively. In the case of dried wolfberries and grapes, the log reduction was achieved by 0.27-log and 0.34-log.

Impact of gaseous ozone to viral substitutes on packaging material surface

To understand the reduction of above viral substitutes (φ6, φX174 and MS2) on three different food packaging matrices, paired comparisons of viral types or material types were tested. Detailed analysis was operated after measurement of the two treatment factors either sample type or microorganism type

(Fig.8,9,10). Fig.8 and 10 showed the detailed analysis results in terms of alternative virus types and suggest a dramatically higher log reduction for φ6 and MS2 on glass and plastic plates compared to φX174. Generally, irrespective of the microorganism type or packaging materials type, the reduction achieved > 0.83-log of microorganisms and inactivation > 84.44% when exposed to 200 mg/h of gaseous ozone. Meanwhile, the data also indicated that log reduction on φ6 by ozone treatment was generally higher compared to other viral substitutes and significantly higher than φX174 on glass plate. Although the reduction of φX174 on kraft paper was more than 1-log, a less than encouraging reproducibility of this finding.

Discussion

Currently, the spread of the COVID-19 pandemic has been highly associated with the food industry. For example, an outbreak occurred at the Beijing Xinfadi Wet Market has triggered the idea that food products, particularly cold-chain foods, can be used as carriers.[21] There is thus an abundant room for further progress in determining new inactivation processes used on foods and packaging materials.

Firstly, our study investigated log reduction and inactivation of three model viruses caused by gaseous ozone in water and gas phase. The results showed φ6, φX174 and MS2 were completely disinfected by ozone in the water phase requiring 3, 5, and 6 min, respectively. Although a slight decrease was observed regarding the reduction and inactivation of the three virus surrogates in gas phase at the same treatment time, there was still a 99% inactivation (reduction ≥ 4-log). Similarly, with the aim of achieving reduction ≥ 4-log on plastic or mental, the required aqueous ozone at 4 ppm was 1.5–1.8 times higher than gaseous ozone for manure-based pathogens.[22] This result can be explained by the fact that ozone could generate more free radicals in the water phase.[23,24] Nevertheless, a major advantage of gaseous ozone is that a flexibility combined with a number of unit operations. Hence, the ozone was selected for follow-up test in gas phase, rather than water phase. In addition, the different treatment time achieved ≥ 4-log for φ6, φX174, and MS2 reported here support the earlier finding that the φ6 was lower resistant to ozone than φX174 and MS2.[25] According to previous studies,[26] it was indicated that ozone could simultaneously attack the nucleic acid and virus capsid causing virus inactivation. Therefore, it is believed that different protein structures and nucleic acids on the surface of the virus may lead to discrepancies in disinfection efficiency.

Secondly, the reduction and inactivation of three model virues on food surface were investigated. Our 1.16-log reduction for MS2 only on soybeans was analogous to the 3.3-log reduction for MS2 on fresh strawbeeries observed by Zhou et al.[27] Another unanticipated finding was that φ6, φX174 and MS2 showed high resistance to gaseous ozone applied to the surface of peanut kernel. A protective effect of the food matrix could most likely be responsible for this low disinfection, as φ6, φX174, and MS2 in water or gas phase were found to be greatly disinfected in our previous tests. There was a little decrease in the disinfection of φ6, φX174 and MS2, which could be attributed to the matrix affected viruses adsorption. Brié et al.[28] found that the low inactivation rate may be due to a protective effect caused by the food

substrate, as HAV proved to pose a high susceptibility in water, with reduced infectivity of ≈ 4-log at short-term ozone processing than on the raspberries tested in their study (at HAV-contaminated).[29,30] The pH may be an another explanation, as peanut kernels are acid matrices compared to soybeans and wolfberries. As reported in literature, MNV-1 was not disinfected in the presence of an acidic pH resembling that of peanut kernels.[31] An alternative hypothesis might invovle the combination of peanut kernels and ozone act synergistically on virus.

It is now well established from a variety of studies that viruses can survive on packaging surfaces including coatings, over packs and fillers.[32,33] A key part of disinfection is the handling of packaging materials. We evaluated the effect of materials on log reduction and inactivation by exposing virus substitution-contaminated packaging materials to 200 mg/h of ozone for 0–6 minutes. In our case, following the change of carrier, a significant difference ($P < 0.05$) in the reduction or inactivation of either pseudovirus was recorded. What we know about the reasons for this phenomenon is largely based on an observational study.[34] Hence, we boldly hypothesize that the inactivation and reduction of the pseudoviruses related to the material surface-functional groups affecting the internal mass transfer of ozone. Surprisingly, φX174 reduced at least 1-log reduction on the Kraft surface compared to the expected 0.5-1 log, whereas a less than encouraging reproducibility of this finding. There is, therefore, a definite need for further studies to effectively determine the effect of ozone on pseudoviruses, particularly on φX174.

Conclusion

The virus crisis has generated disruption and setbacks in food industry, resulting in a new change of disinfection technologies that the world will have to face. We thus aim to contribute to this growing area of research by exploring the role of gaseous ozone in the disinfection of food and packaging materials. Statistical analysis revealed that ozone was generally not appropriately applied on the surface of peanut kernel and dried wolfberry for inactivating MS2. φ6 showed promise as it was greatly disinfected on most foods and packaging materials, but new test conditions appear necessary in order to achieve a significant log reduction on peanut kernel. Ozone treatment is effective when reducing φX174 on foods and packaging materials, as expected, peanut kernel decreased its efficacy for inactivating φX174, while kraft paper did not protect the virus from inactivation as expected instead enhancing its inactivity. For this project, kraft paper might not be the most proper packaging material to simulate the real conditions encountered owing to a less than encouraging reproducibility of this result. To boost the application of gaseous ozone in future industries, it would therefore be worthwhile to study the survival or disinfection of foodborne pathogens or their substitutes in batches of different foods and packaging materials exposed to ozone.

Declarations

Author's Contribution

KH and HL: Investigation, Data analysis, Writing original draft. JZ: Investigation, Data analysis. YH: Writing review. JC and JH: Supervision, Writing original draft, Writing review and editing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable, because this manuscript does not contain any studies with human or animal subjects.

Conflict of Interest

The authors declare that there is no competing financial interests in this work.

Availability of Data and Materials

The data and materials during the current study are available from the corresponding author.

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Tables

Table 1. Microbiological parameters of bacteria and phages used

Bacterial or viral strains	Characteristics	Growing conditions	Bacterial host
E. coli ATCC15597	-	TSB, 37˚C, 200 rpm	-
E. coli ATCC13706	-	TSB, 37˚C, 200 rpm	-
P.svringe NBRC14084	-	TSB, 26˚C, 100 rpm	-
PhageMS2	25nm,nonenveloped,linearssRNA, Icosahedron shape	-	*E. coli* ATCC15597
PhageφX174	25-27nm,nonenveloped,linearssDNA, Icosahedron shape	-	*E. coli* ATCC13706
Phageφ6	85nm,enveloped,segmented dsRNA, Spherical shape	-	*P.svringe* NBRC14084

Table 2. Preparation of foods and packaging materials

Materials	Weight or area	Inoculation results		
		φ6	φX174	MS2
Dried wolfberry	0.50g±0.02^{a}□g□	5.29±0.11^{b}	6.02±0.12^{d}	6.19±0.02^{b}
Fresh wolfberry	2.50g±0.05^{d}□g□	5.45±0.13^{b}	5.50±0.08^{b}	6.24±0.04^{b}
Grapes	5.78±0.20^{e}□g□	5.69±0.09^{c}	5.93±0.20c	5.49±0.07^{a}
Peanut kernels	1.50±0.20^{c}□g □	4.27±0.06a	4.90±0.00^{a}	7.33±0.12^{c}
Soya beans	1.05±0.02^{b}□g□	6.38±0.07^{d}	6.00±0.12^{d}	6.25±0.10^{b}
Plastic panels	0.8×0.8^{a}□cm□	5.04±0.08^{b}	5.73±0.18^{b}	6.58±0.07^{b}
Kraft paper	0.8×0.8^{a}□cm□	4.71±0.16^{a}	5.89±0.12^{b}	6.26±0.03^{a}
Glass panels	1.8×1.8^{b}□cm□	4.72±0.11^{a}	5.05±0.17a	6.25±0.10^{a}

Inoculation results represent log values of virus titers for contaminated foods and packaging materials

Figures

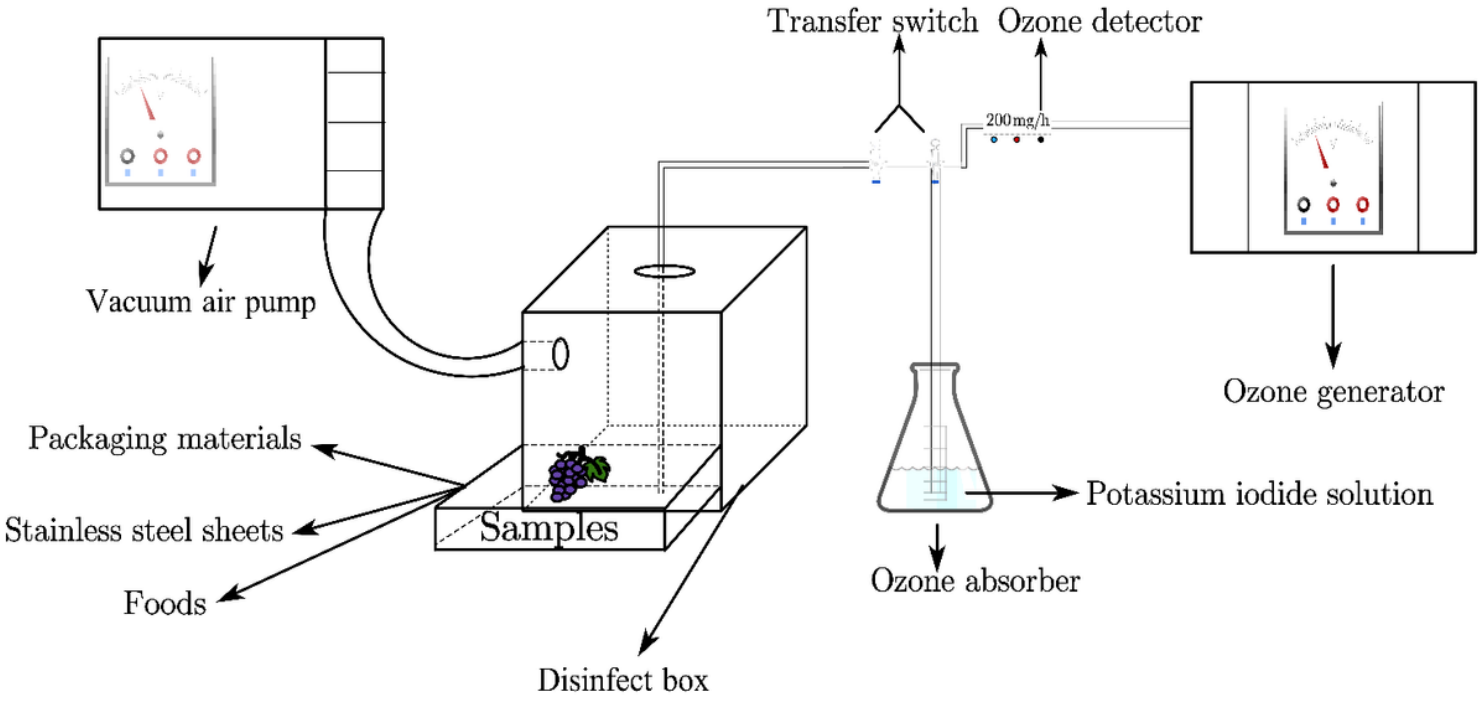

Figure 1

A schematic diagram of the experimental set-up of ozone treatment.

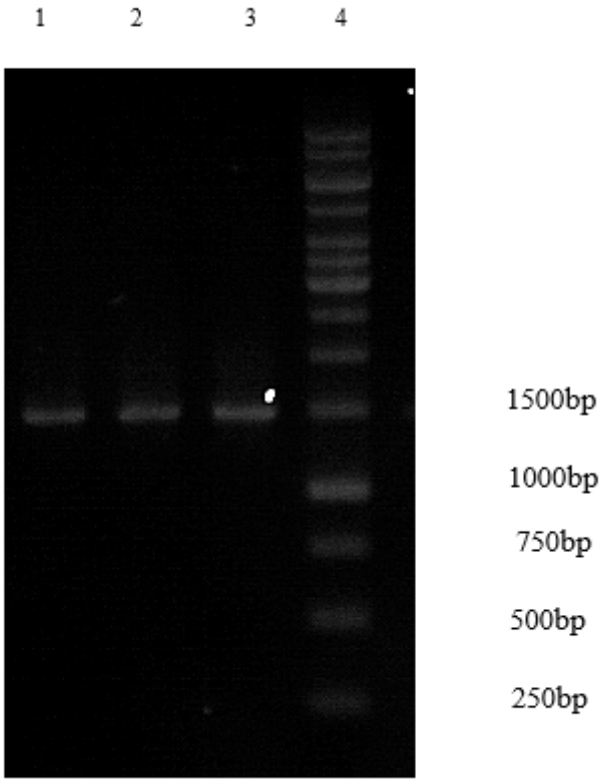

Figure 2

Agarose gel electrophoresis of amplified products：1:*P.svringe*.NBRC14084 16S-rRNA；2：*E.coli*.ATCC 13706 16S-rRNA；3：*E.coli*.ATCC 15597 16S-rRNA 4:marker；

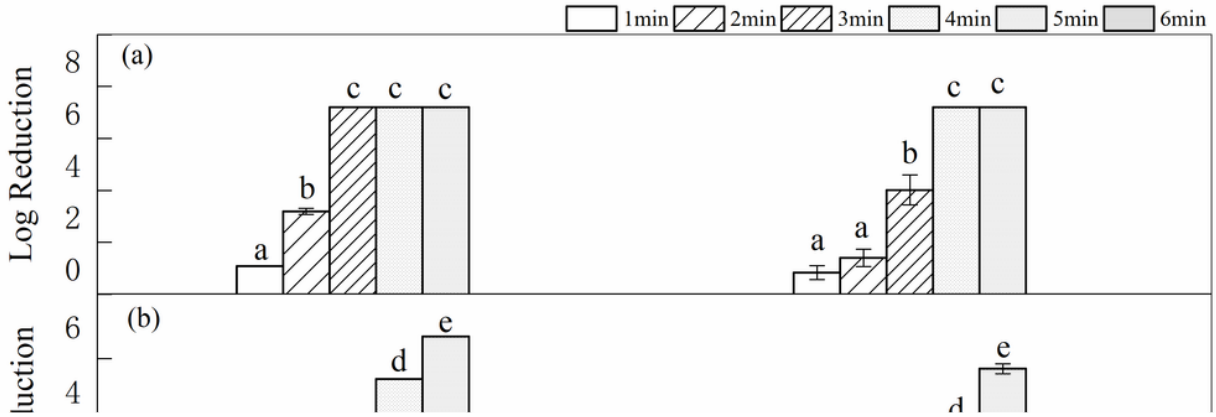

Figure 3

Impact of gaseous ozone treatment time on log reduction of viral substitutes in water, gas phase. (a) φ6 (b) φX174 (c) MS2

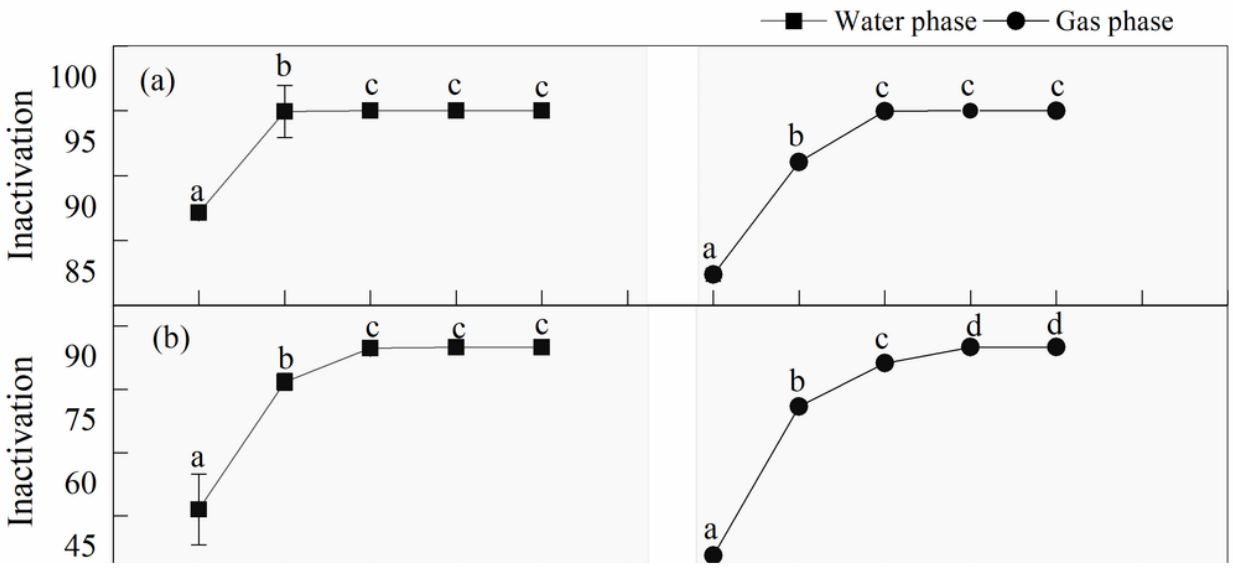

Figure 4

Impact of gaseous ozone treatment time on inactivation of viral substitutes in water, gas phase. (a) φ6 (b) φX174 (c) MS2

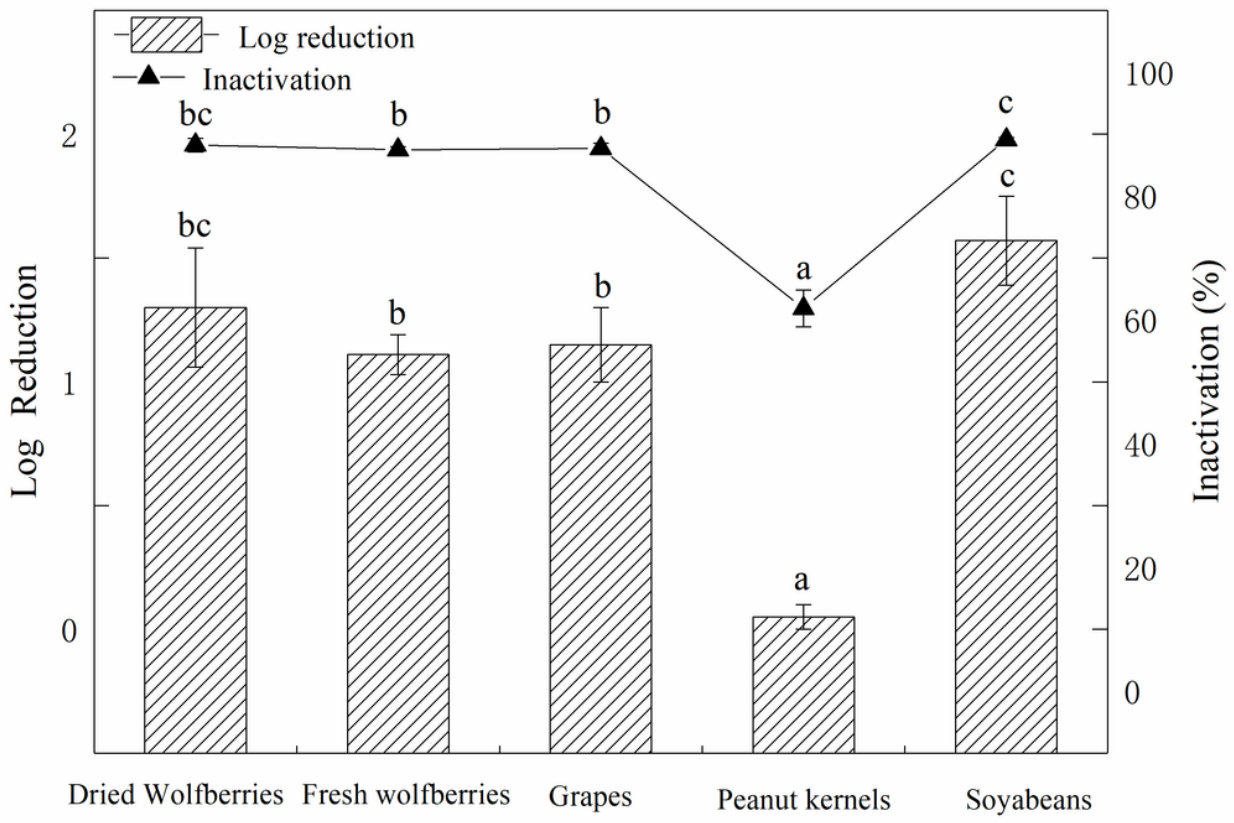

Figure 5

Impact of the ozone gas treatment on log reduction and inactivation of φ6 on foods

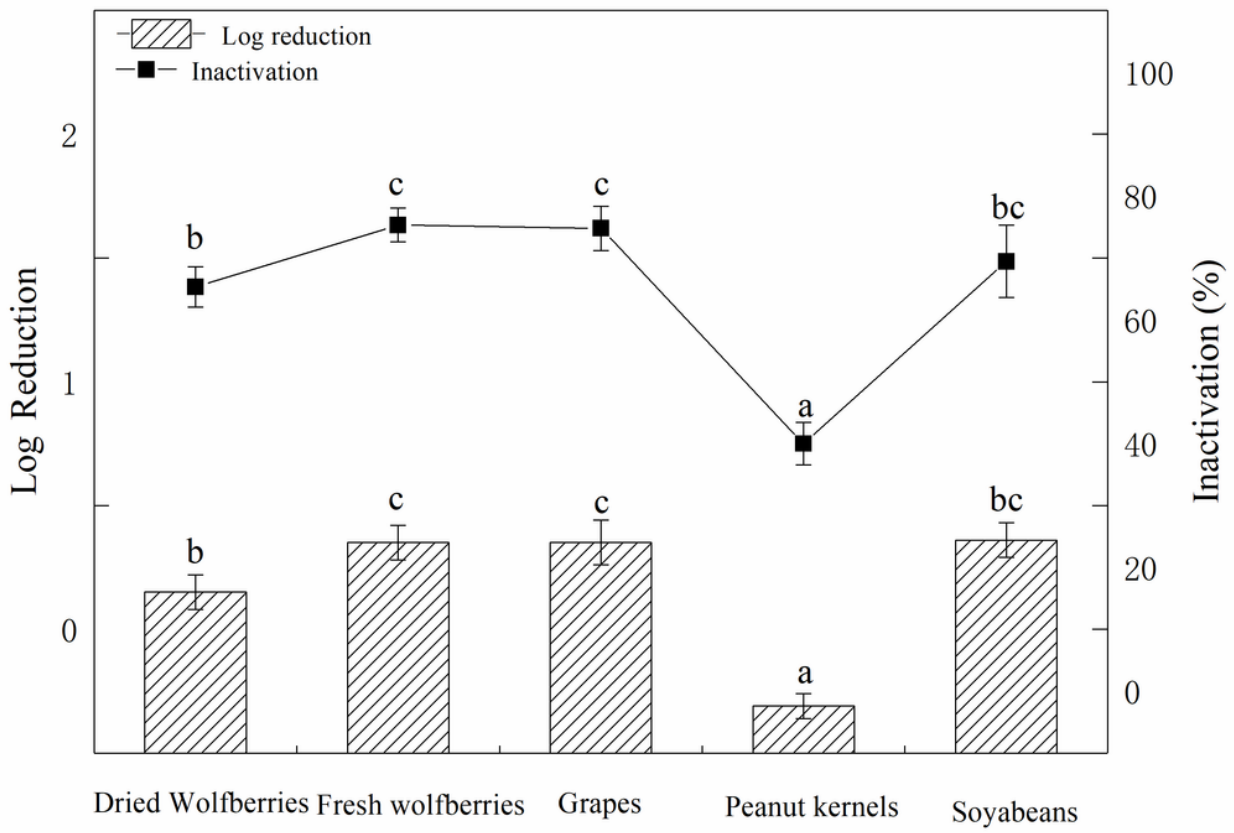

Figure 6

Impact of the ozone gas treatment on log reduction and elimination rate of φX174 on foods

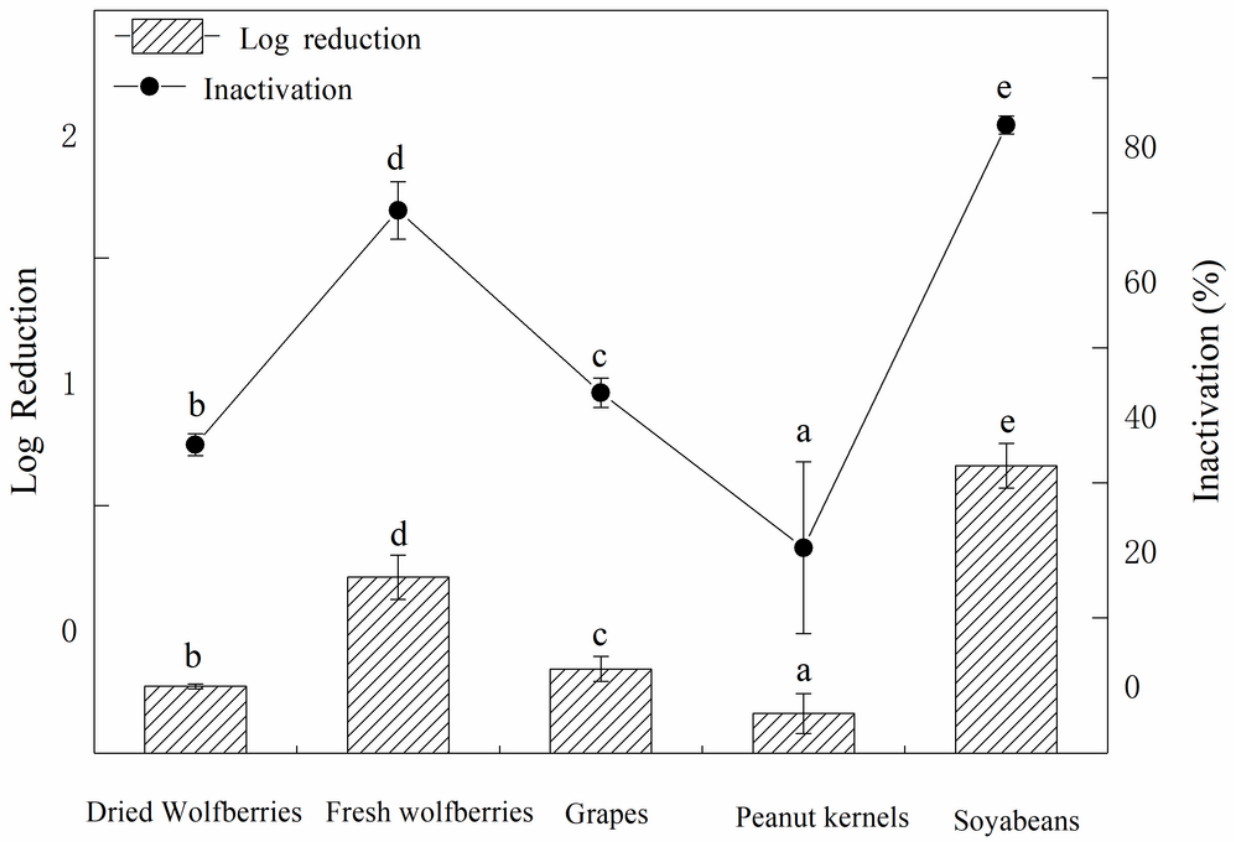

Figure 7

Impact of the ozone gas treatment on log reduction and inactivation of MS2 on foods

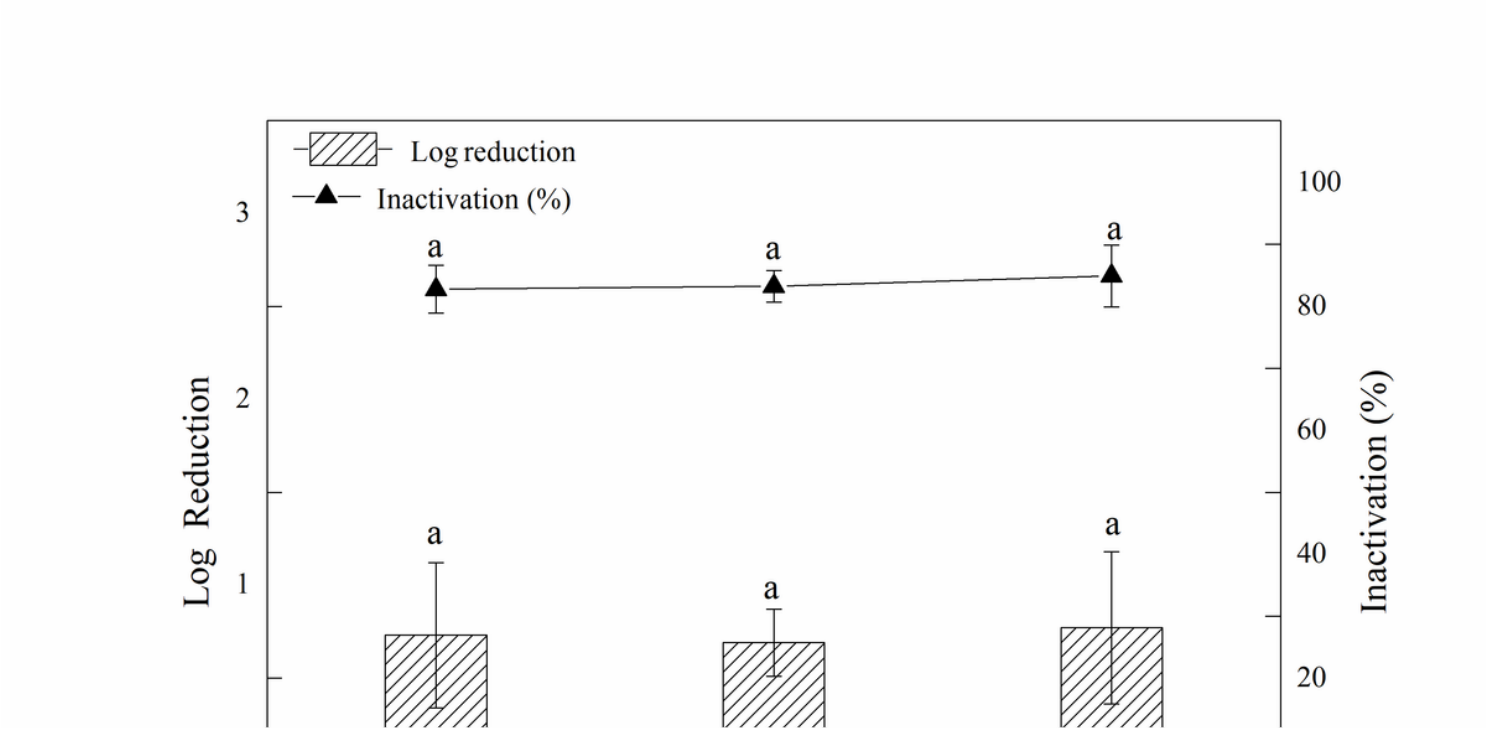

Figure 8

Impact of the ozone gas treatment on log reduction and inactivation of φ6 on packaging materials

Figure 9

Impact of the ozone gas treatment on log reduction and inactivation of φX174 on packaging materials

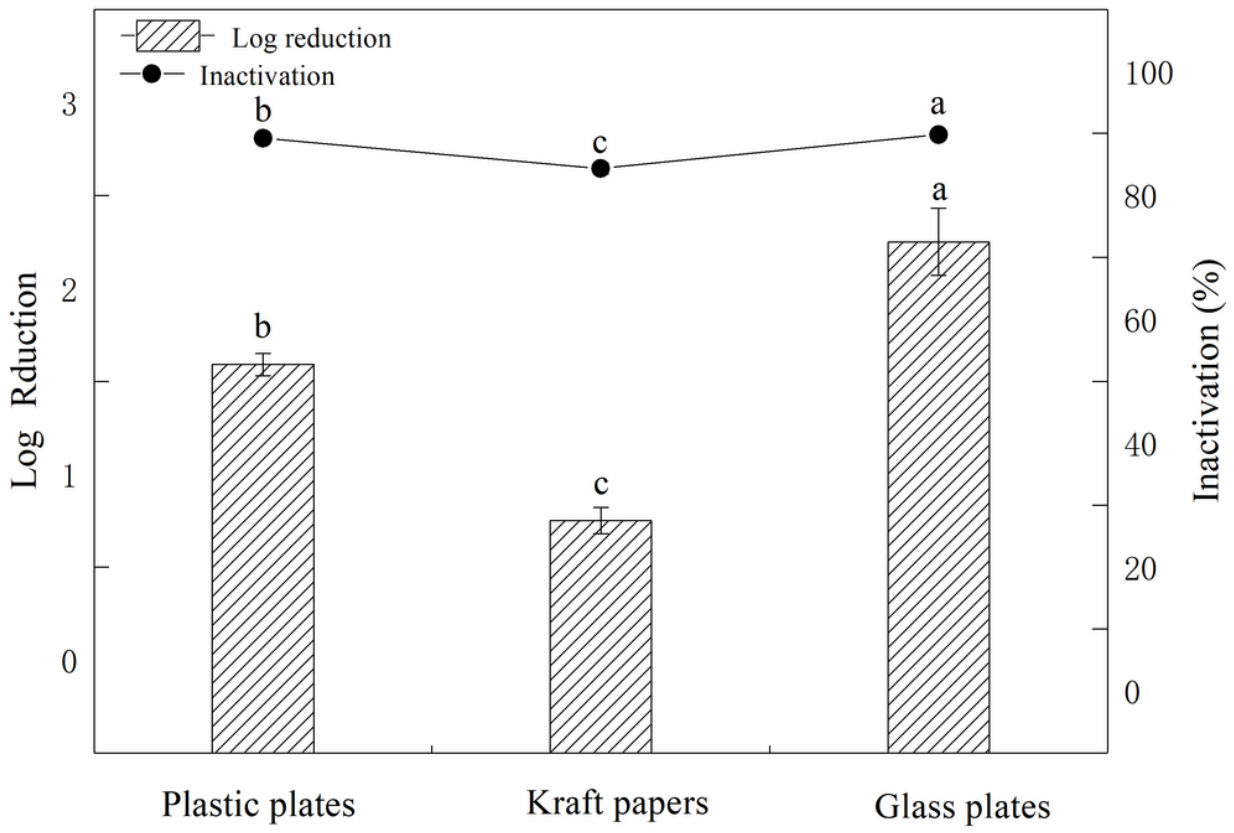

Figure 10

Impact of the ozone gas treatment on log reduction and inactivation of MS2 on packaging materials