The possibilities of using Ultrasonically Activated Streams to reduce the risk of foodborne infection from salad

Authors: Weng Yee Chong [a], Thomas J. Secker [b], Craig N. Dolder [a,c], Charles W. Keevil [b] and Timothy G. Leighton [a,c,*]

[a] Institute of Sound and Vibration Research, University of Southampton, Southampton, SO17 1BJ, UK

[b] Environmental Healthcare Unit, School of Biological Sciences, University of Southampton, Southampton, SO17 1BJ, UK

[c] Sloan Water Technology Ltd., 1 Venture Road, Chilworth, Southampton, SO16 7NP, UK

**corresponding author*

Postal Address: Institute of Sound and Vibration Research, University of Southampton, Southampton, SO17 1BJ,

Telephone: +44 23 80 592291

E-mail address: tgl@soton.ac.uk

Abstract

In this study, the effects of an Ultrasonically Activated Stream (UAS) on the removal of microbial contaminants from spinach leaves were investigated. The microbial loads on samples cleaned with UAS and non-UAS were enumerated using the cell culture method and were compared against unwashed samples on day 0 and day 6 post cleaning. The effects of UAS cleaning on leaf quality were also examined through both macroscopic and microscopic inspection as well as electrolyte leakage rate measurement. Results showed that the microbial load on samples cleaned with UAS for 2 minutes on day 6 post cleaning was significantly lower than those treated without ultrasound. Comparison between the cleaning effects of UAS for 40 seconds and 2 minutes indicated that a cleaning duration of 2 minutes allowed sufficient time for UAS to disaggregate and detach the microbial contamination more effectively. In this case, the induction of bacteria into viable but nonculturable state does not affect the shelf-life test results as much as that of a 40-second clean. UAS cleaning for 2 minutes did not produce significant surface damage that can affect the overall leaf quality. These findings highlighted the potential of UAS systems in the salad industry to improve the microbiological quality and shelf-life of salads.

Keywords

Ultrasound, Bubbles, Ultrasonic cleaning, Food safety, Foodborne diseases, Ready-to-eat salads.

Introduction

Human health is intimately linked with good nutrition, for example in the avoidance of food poisoning and malnutrition. Studies have shown that a high consumption of vegetables, especially vegetables eaten raw, i.e. salads, can potentially reduce the risk of cardiovascular diseases, Type II diabetes and certain types of cancer (Fabbrin and Crosby 2016). However, ready-to-eat (RTE) raw products, such as salads, are prone to microbial contamination from farm to fork and the consumption of food contaminated with foodborne pathogens such as *E.coli O157:H7*, *Salmonella spp, Listeria monocytogenes* can lead to foodborne diseases which can cause hospitalisations and may be fatal in vulnerable groups (Holland et al. 2020). RTE salads have also been reported as one of the common vehicles of foodborne pathogens that led to numerous outbreaks of foodborne diseases (Machado-Moreira et al. 2019). Increased knowledge of the characteristics of pathogens on fresh produce has not translated into fewer outbreaks. Outbreaks associated with fresh produce increased from fewer than 20 in the 1970s, to more than 100 in the 1990s (Sivapalasingam et al. 2004). There were 5 reported deaths when 210 people were infected across Canada and 35 US states (96 hospitalized, including 27 who developed haemolytic uremic syndrome) with the O157:H7 strain of *E. coli* on Romaine lettuce from the Yuma growing region, in an outbreak lasting from March to June 2018 (Centers for Disease Control and Prevention [CDC] 2018). Despite this, in the following year (September 2019-January 2020) an outbreak of the O157:H7 strain of *E. coli* on Romaine lettuce from the Salinas Valley growing region in California infected 167 people across 27 states (with 85 hospitalizations including 15 people who developed haemolytic uremic syndrome) (CDC 2020).

It is also of concern that in the 2018 outbreak, genes for antibiotic resistance to chloramphenicol, streptomycin, sulfisoxazole, tetracycline, and trimethoprim-sulfamethoxazole were found in isolates from 184 ill people identified (CDC 2018). Genes for antibiotic resistance to ampicillin and ceftriaxone were also found in isolates from four of those ill people (CDC 2018). In the 2019 outbreak, bacterial isolates containing antibiotic resistance

genes were obtained from 2 of the 159 patients studied, one for ampicillin, and one with combined resistance for ampicillin, chloramphenicol, streptomycin, sulfisoxazole, and tetracycline (CDC 2020). Therefore, it is crucial to investigate ways to improve the microbiological safety of RTE salads to reduce the risk of foodborne diseases. This paper presents the first evidence that Ultrasonically-Activated Streams are worth investigation towards this goal.

In the salad processing line, 'cold-water' washing (i.e. without heating the water) is the main process for the removal or reduction of dirt and microbiological contaminants (Luo 2007, Bilek and Turantas 2013). Chemical disinfectants such as chlorine-based compounds have been widely used to sanitise contaminated wash water to prevent cross contamination (Gil et al. 2009, Joshi et al. 2013). However, there are limitations in the volume and concentration of chemical disinfectants that can be used in the washing process, as by-products could lead to health deterioration and environmental issues (Gil et al. 2009). Furthermore, chemical disinfectants can affect the nutritional value (Gil et al. 2009), texture and flavour of RTE salads (Koukkidis et al. 2017). Another limitation of chemical disinfectants for RTE salad washing is that food borne pathogens can enter a viable but nonculturable (VBNC) state when exposed to chemical disinfectants (Afari and Hung 2018, Highmore et al. 2018). VBNC bacteria pose a serious threat to food safety as they remain undetected using standard cell culture techniques but still exhibit virulence that can lead to foodborne diseases (Afari and Hung 2018, Highmore et al. 2018). Therefore, it is crucial to seek chemical-free alternatives for RTE salad cleaning without compromising leaf quality and shelf-life.

Ultrasound has been used in cleaning for more than 70 years in the form of ultrasonic cleaning baths (Leighton et al. 2005, Mason 2015), where high power ultrasound induces inertial cavitation in the liquid to generate the cleaning effect (Mason et al. 1996, Leighton et al. 2013). During inertial cavitation, bubbles undergo sudden explosive expansion followed by a much more rapid collapse with a bubble wall that accelerates as the bubble size reduces (Leighton

et al. 2000), generating extreme localised temperatures and pressures (Leighton et al. 1997, Suslick et al. 1999, Bilek and Turantas 2013). Such inertial collapse leads to the formation of shock waves and collapsing bubbles can also involute to form high speed liquid jets (Jamaluddin et al. 2011) that can dislodge contaminants as they impinge on the contaminated surface (Awad and Nagarajan 2010). This process offers effective detachment and can inactivate or rupture bacterial cells and biofilms (Declerck et al. 2010, Bilek and Turantas 2013, Vyas et al. 2019). Such an effect can also enhance the efficiency of bactericides and chemical disinfectants in the inactivation/destruction of pathogens and biofilms (Seymour et al. 2002, Lattwein et al. 2020). However, the effects arising from inertial cavitation may also cause damage to object surfaces, even metals (Awad and Nagarajan 2010, Turangan et al. 2017, Yasui 2018). Another effect resulting from the rapid collapse is the generation of free radicals (Leighton 1994, Birkin et al. 2001) that can react to form antimicrobial compounds such as hydrogen peroxide (H_2O_2) (Birkin et al. 2001, Yasui 2018). Free radicals and H_2O_2 can damage healthy cells and produce off-flavours in foods (Donnelly and Robinson 1995, Chen et al. 2020). Another major drawback of ultrasonic baths for fresh produce cleaning, is that food items are prone to cross contamination as bacteria removed from the surface will remain in the wash water (Seymour et al. 2002). The problem of cross contamination within an ultrasonic bath can be alleviated using cavitation intensifying bags (Van Zwieten et al. 2017), however, this might not be a feasible method for large scale processing of fresh produce.

An alternative ultrasonic cleaning technology, the ultrasonically activated stream (UAS), eradicates the issue of cross-contamination by incorporating the ultrasound in a rinse (Leighton 2014). In contrast to an ultrasonic bath, UAS allows items to be cleaned in place, obviating the need to immerse the object to be cleaned in a bath or tank (Leighton 2015), allowing items as large as railway track to be treated in place (Goodes et al. 2016). A distinctive characteristic of UAS devices is that they do not produce inertial cavitation (Leighton 2015). In UAS devices, ultrasound is used to generate Faraday waves (Fig. 1) and other surface waves on the walls of the bubbles (Faraday 1831, Maksimov and Leighton 2001, Leighton

2004, Maksimov and Leighton 2011), generating convection and shear forces in the surrounding liquid (Maksimov and Leighton 2018). As the stream is projected onto the contaminated surface, bubbles are attracted to the surface by Bjerknes radiation forces and into crevices by secondary Bjerknes radiation forces (Maksimov and Leighton 2018). This will allow the convection and shear forces to scrub contaminants thoroughly from the surface (Leighton 2015). The effectiveness of UAS in the removal of both non-biological and biological contaminant and biofilms from various surfaces have been demonstrated in previous controlled studies (Birkin et al. 2015, Howlin et al. 2015, Goodes et al. 2016, Salta et al. 2016, Malakoutikhah et al. 2020, Secker et al. 2020, Chong et al. 2021).

Salad leaves are highly susceptible to structural damage during the processing stage (Ariffin et al. 2017) and such damage can cause a negative impact on the nutritional, organoleptic and microbiological qualities (Cocetta et al. 2014, Ariffin et al. 2017). Studies have shown that foodborne pathogens have a higher tendency to attach onto cut edges on the leaf surface (Seo and Frank 1999). Damaged leaves can promote the survival and proliferation of foodborne pathogens (Harris et al. 2003) as wounds on the surface provide an entry point for bacteria to internalise into the subsurface regions (Li et al. 2008, Kroupitski et al. 2009). This will provide protection for pathogens against washing and sanitising treatments (Seo and Frank 1999, Millan-Sango et al. 2015). Therefore, it is crucial to ensure that the cleaning methods applied to salad leaves will not produce any form of surface damage.

The aim of this study was to investigate the potential of ultrasonic cleaning using non-inertial cavitation in improving the microbiological safety of RTE salads as a measure to reduce the risk of foodborne illness. This was performed by evaluating the efficacy of UAS in the removal of microbiological contaminants from salad leaves without causing any significant damage to the leaf surface. The cleaning performance of UAS was compared to that of a water rinse with the same flow rate and flow velocity without ultrasonic activation (non-UAS). Another objective of this study was to determine whether UAS treatment will improve the shelf-life of salad leaves

and this was done by comparing the total viable count (TVC) of the leaf samples on day 0 and day 6 post cleaning. Treated leaf samples were examined for presence of surface damage through visual and microscopic inspection and measuring the electrolyte leakage of the leaves after treatment.

Materials and methods

Leaf samples

Spinach leaf samples were supplied by Vitacress Salads Ltd and used within 48 hours of arrival. The samples were kept refrigerated at 4 ± 1 ^{o}C when not in use. Leaf cleaning experiments were carried out using leaf discs and whole leaves. The purpose of the leaf disc cleaning experiment was to investigate the minimum washing duration required to produce significant effects from UAS cleaning, whereas the purpose of the whole leaf cleaning experiment was to produce a cleaning condition closer to standard cleaning/rinsing procedures.

Leaf discs were prepared using a sterile 8.75 mm cork borer (Breckland Scientific, UK). The cork borer was disinfected with 70% ethanol between every sample. The whole leaf cleaning experiment was conducted in duplicate with three different batches of spinach leaves. It was important to use commercial produce to get a realistic environmental microbial load and leaves representative of that which the industry must manage. Three different batches were used because the duration of the experiment lasted over more than a single growing season for commercial produce, and so as the year progressed, and each country ended its season, the supply moved. Details of the sources of the three batches are given in the Supplementary Data Table S1. Vitacress Salads Ltd., who supply the leaf, have strict quality control to assure the standard of produce is comparable. In order to reduce variability within the same batch of leaves, leaves with even distribution of sizes and minimal pre-existing injuries were chosen.

Experimental setup and cleaning procedures

The experimental setup was as shown in Fig. 2. A Mark 2 StarStream UAS device (Ultrawave Ltd., Cardiff, Morgannwg, Wales) was connected to a non-recirculating water supply system equipped with a high capacity water filter kit (Best Water Technology, UK) and an E.Sybox Mini water pump (Dab Water Technology, Italy). Tap water was used in the system and the water temperature was measured to be 24 ± 1 °C throughout the experiment. The UAS device produces a gentle stream of water at a flow speed on 2.0 ± 0.1 L/min in a stream 1 cm in diameter as it leaves the nozzle. The electrical supply connected to the ultrasonic transducer (Fig. 2) was designed to generate 135 kHz ultrasound with acoustic pressure amplitude that is sufficiently high to produce cleaning effects arising from non-inertial cavitation, but remain well below the Blake threshold for inertial cavitation. The power consumption of the UAS device was 81.26 ± 1.65 W (this was measured using a Brennenstuhl PM231 single-phase plug-in energy meter with ± 1 % accuracy). At 135 kHz, the zero to peak acoustic pressure amplitude required to generate inertial cavitation over a range that exceeds the maximum and minimum bubble sizes that could be present (1-1000 microns radius) ranges from 140 to 200 kPa. The acoustic pressure of the UAS was measured using a hydrophone (Reson TC4038) placed within the stream, with its acoustic centre 5 mm from the point where the water exits the nozzle. The hydrophone (Reson TC4038) used to obtain these measurements was factory-calibrated from 50 kHz to 800 kHz. To monitor the consistency of the acoustic performance of UAS over time, hydrophone measurements were acquired at every minute for up to 20 minutes, and a typical variation is plotted in Fig. 3. The pressure amplitude presented in Fig. 3 were calculated by multiplying the rms value of the hydrophone signal with the hydrophone sensitivity (-229.5 dB re 1 V /µPa) at around the frequency of excitation, i.e. 135 kHz. (*Note: As hydrophones are calibrated for free field conditions, in this case where the hydrophone was placed within the stream, the calibration is only valid as a relative measure to allow any future users to replicate the experimental conditions)*. When this electrical signal

was turned off, the stream of water was identical except that no ultrasound was present (this was used to provide the 'non-UAS' cleaning condition that was compared to the UAS cleaning).

The bubbles required for cleaning are naturally present in the water supply, a steady-state population of bubbles being built up from the filling of the reservoir with tap water that naturally contains air bubbles, then then pumping it through the pipework and past an ultrasonic source (Fig. 2). Demonstrated in Fig. 1, the bubbles just before (panel (a)) and just after (panel (b)) the ultrasound was turned off. Bubbles (i) and (ii) have diameters of around 250 µm and 280 µm respectively, small enough to host Faraday waves according to theory (Maksimov and Leighton, 2001), but bubble (iii) is too large to host them, at around 350 µm diameter. Therefore, as the liquid circulated around the system, a filter was placed in the flow that removed bubbles having diameters greater than 300 µm.

The leaf samples were placed on a strainer plate, which was positioned below the device nozzle. The distance between the device nozzle and the leaf surface was set at 1.0 ± 0.2 cm as 1.0 cm is the optimum distance which the cleaning performance of the device is most consistent. The exposure condition of leaf samples was characterised using a contact time index, which can be calculated using Eq. 1, where $Area_{UAS}$ represents the area covered by UAS during cleaning; $Area_{sample}$ represents the area of the leaf disc or whole leaf; T represents the total cleaning duration per sample. The contact time indexes for leaf discs and whole leaves are shown in Table 1.

$$Contact\ time\ index = \frac{Area_{UAS}}{Area_{sample}} \times T \qquad \text{(Eq.1)}$$

For leaf disc cleaning, the cleaning duration was set at 10 seconds and 20 seconds per side. After cleaning, the leaf discs were transferred to Eppendorf tubes. For whole leaf cleaning, five grams of spinach leaves were cleaned (one leaf at a time) for 40 seconds (per leaf, 20 seconds each side) with UAS and another five grams were cleaned with non-UAS, as

described previously. After cleaning, the leaves were spun dry with a salad spinner (OXO, UK) for 10 minutes. The salad spinner was disinfected with 70% ethanol between every cleaning treatment. This was repeated with a cleaning duration of 2 minutes (per leaf, 60 seconds each side).

A shorter test exposure was chosen for the leaf disc tests, compared to that used in the whole leaf cleaning test, because the exposure of a given region of microbe to the stream, depends on the exposure of a given area equal to (or less than) the cross-section of the stream, not the duration of the test. Given that the stream is smaller than a leaf, it would make no sense to give a disc (which is in the stream throughout the treatment time) and a leaf (each part of which is only covered by the stream for part of the treatment time) identical treatment times.

Microbiological examination

For the leaf disc cleaning experiment, the microbial suspension for each sample was prepared by adding 1 ml sterile maximum recovery diluent (MRD) (Sigma Aldrich, UK) into Eppendorf tubes that contained four leaf discs. The microbial suspension was incubated at room temperature for 15 minutes before being spun at 3000 rpm using a vortex mixer (Classic Vortex Mixer, Fisherbrand®, UK). The solution was serial diluted and plated onto nutrient agar plates in volumes of 20 µL in triplicates. The plates were incubated at 37 ^{o}C for 24 hours.

For the whole leaf cleaning experiment, five grams of leaves were added into a Stomacher® blender bag containing 45 ml sterile MRD (Sigma Aldrich, UK). The Stomacher® bag was pulsified with a Pulsifier (Pulsifier II ®, Microgen Bioproducts, UK) for 1 minute. The microbial suspension was serially diluted by adding 1 ml of the suspension into 9 ml of maximum recovery diluent before being spread-plated in volumes of 100-200 µL on triplicate nutrient agar (Sigma Aldrich, UK) plates. The plates were incubated at 37 ^{o}C for 48 hours. To improve data reliability, only plates that had 30 - 300 colonies were chosen. The microbial load of the samples was expressed in units of log colony forming units per gram (CFU/g).

Shelf-life analysis using TVC

Whole leaf samples were kept in resealable bags and stored at 4 ± 1 ^{o}C up to day 6 post cleaning. Macroscopic images of the samples were acquired on day 0 and day 6 post cleaning (Supplementary Fig. S2). On day 6, the samples were examined using the procedures described in the previous section. The whole leaf cleaning experiment was performed in duplicate and was repeated three times, each time with a fresh batch of spinach leaves. In this experiment, it is hypothesised that samples with lower TVC on day 6 will have a longer shelf-life.

Detection of surface damage

The presence of surface damage on the leaves was examined using visual inspection at both macroscopic and microscopic levels. For macroscopic inspection, the adaxial and abaxial surfaces of the leaf samples were visually examined for the presence of any form of surface damage before and after cleaning. Apart from visual *in situ* examination, macroscopic images of the samples were also acquired using a camera (Nikon D800) before and after cleaning (Supplementary Fig. S3).

For microscopic inspection, the leaf samples were examined using an Episcopic Differential Interference Contrast (EDIC) microscope (Nikon Eclipse, LV100, custom modified by Best Scientific UK (Keevil, 1992)) at a total magnification of x100 and x400. Microscopic images were acquired using ImagePro plus software (Media Cybernetics). The microscopic image resolutions are 0.96 µm/pixel for x100 magnification and 0.24 µm/pixel for x400 magnification. To avoid imaging naturally pre-existing wounds on the leaves, leaf discs were excised from whole leaf samples before being transferred onto the microscope stage for image acquisition. To assist evaluation of surface damage, microscopic images were taken of ‘damaged control’ samples, which contained wounds that were manually created using a pair of stainless steel tweezers to handle the leaves in a normal manner, with normal manual pressure (Supplementary Fig. S4). These ‘damaged controls’ were acquired for comparison purposes

with the normal controls, and the washed samples. For each leaf disc, microscopic images were acquired at 9 positions as indicated in Fig. 4 (a). At each position, images were acquired at 10 focal positions and the distance between the top and bottom focal positions, ΔZ was measured. The difference in ΔZ for micrographs showing uneven surface topography, wounded regions at both x100 and x400 magnification levels are shown in Fig. 4(b). The images were processed using an ImageJ plugin (Plugin name: Extended Depth of Field (Forster et al., 2004)) that merges a stack of images acquired at varying focal positions to render into a composite image.

Leaf quality evaluation using electrolyte leakage rate

Electrolyte leakage measurements were performed on whole leaf samples on day 0 and day 6 post-cleaning to investigate the effect of UAS cleaning on leaf quality over time. Each leaf sample was incubated in 50 ml of sterile Milli-Q water for 60 minutes. The initial and final conductivity (C_0 and C_1) of the solution was measured using a conductivity meter (Hanna Instruments, UK, Model number: HI99300) after incubating for 1 minute and 60 minutes. The solution was then autoclaved at 121.5 ^{o}C for 15 minutes. The total conductivity (C_T) of the solution was measured when the solution had cooled to room temperature. The electrolyte leakage was calculated using Eq. 2 (Iakimova and Woltering 2015).

$$Electrolyte\ leakage = \frac{C_1 - C_0}{C_T} \times 100\% \qquad \text{(Eq.2)}$$

Statistical analysis

The statistical analysis for the cleaning results was performed using SigmaPlot Version 13.0 software (Systat Software Inc.). The microbial load remaining on leaf disc and whole leaf samples were analysed using Kruskal-Wallis one-way Analysis of Variance (ANOVA) on ranks, followed by a *post-hoc* Dunnett's Multi Comparison test to compare the cleaned samples against the uncleaned (control) samples. The differences in microbial load between cleaning

289 treatments and durations were evaluated using the Mann-Whitney Rank Sum test. The
290 statistical analysis for the ΔZ and electrolyte leakage measurements were similar except that
291 no *post-hoc* comparison test was carried out for the cases in which there were no significant
292 differences between all sample groups when analysed using ANOVA on ranks.

293 ## Results

294 ### The cleaning efficacy of UAS and non-UAS treatment

295 For leaf disc cleaning, statistical analysis showed that all cleaned leaf discs had significantly
296 lower microbial load than the control group ($P < 0.05$) (Fig. 5). Cleaning with and without UAS
297 for 20 seconds were not significantly different from each other, whereas at a cleaning duration
298 of 40 seconds, cleaning with UAS was shown to remove a significantly higher number of
299 microbes than non-UAS ($P = 0.029$). When comparing the same cleaning method but with
300 different durations, an increased cleaning duration of 10 s each side did not significantly
301 enhance the removal of microbiological contaminant.

302 For whole leaf cleaning, all cleaned samples had significantly lower microbial loads than the
303 control samples ($P < 0.05$) except for samples cleaned with non-UAS for 40 seconds (Fig. 6).
304 When comparing between both treatments at a cleaning duration of 40 seconds, UAS cleaned
305 significantly better than non-UAS ($P = 0.009$), while there was no significant difference when
306 the samples were cleaned for 2 minutes. The effect of increasing cleaning duration was similar
307 to that of the leaf disc cleaning experiment, where no significant improvement was observed.

308 ### Shelf-life test

309 For whole leaf samples which were examined on day 6 post cleaning, only samples cleaned
310 with UAS for 2 minutes had a significantly lower microbial load than the control samples
311 ($P < 0.001$), while other sample groups showed no significant differences when compared
312 against the control samples (Fig. 6). When comparing between treatments, unlike the results

obtained on day 0, there was no significant difference between the microbial loads remaining on samples cleaned with both methods for 40 seconds on day 6 post cleaning. On the contrary, samples cleaned with UAS for 2 minutes had a significantly lower microbial load than those cleaned with non-UAS ($P = 0.041$). Unlike UAS cleaning, when comparing between cleaning durations, there were no significant differences in the samples cleaned with non-UAS. The microbial load on samples cleaned with UAS for 2 minutes was lower than those cleaned for 40 seconds ($P = 0.009$). The visual quality of the samples treated with and without UAS did not deteriorate over 6 days (Supplementary Fig. S2).

Presence of surface damage post cleaning

For all the samples cleaned with and without UAS at both durations, no visible surface damage was detected through macroscopic visual inspection. All cleaning treatments did not cause any defects to the overall visual quality of the leaves (Supplementary Fig. S3).

Microscopic inspection at x100 magnification (Supplementary Fig. S5) allowed a more rapid detection of surface damage as compared to x400, but was insufficient to reveal the degree of surface damage as clearly as compared to x400 magnification (Fig. 7). As demonstrated in Fig. 7, microscopic inspection at x400 magnification revealed slight abrasion on the epidermal cells of the samples irrespective of treatments and cleaning durations. Nonetheless, the visual structure of the epidermal cells remained undamaged, unlike as observed in the damaged control samples, where the epidermal cells were hardly visible in the wound since the overall structure was damaged.

The ΔZ measurements (Fig. 8) were used to quantify the degree of surface damage observed in the micrographs. At both magnification levels, the ΔZ varied between each micrograph because of the uneven and variable topography of the leaf surface, and the mean was greater at the lower magnification because the imaged area covers a larger footprint of the wound (see Fig. 4(b)). The ΔZ for micrographs of wounded regions on the damaged control sample

was higher than all other sample groups because a greater ΔZ was needed to obtain an increased depth of view to visualise both the epidermis and the base of the wound. Unlike the data obtained at x100 magnification, the ΔZ measurements at x400 magnification showed that there were significant differences between the damaged control and all sample groups (undamaged control and all treated samples). When comparing between the undamaged control and the treated samples, results in Fig. 8 indicated that the slight surface abrasion observed in Fig. 7 was not significant.

Electrolyte leakage rate

The electrolyte leakage rates of leaf samples treated with UAS and non-UAS for 40 seconds and 2 minutes on day 0 and day 6 post cleaning were shown in Fig. 9. On day 0, the electrolyte leakage of samples treated with UAS for 2 minutes was the highest and was significantly higher than that of non-UAS for 2 minutes ($P = 0.038$). On day 6, the electrolyte leakage of the samples was lower than that of day 0 post cleaning. Comparison using ANOVA on ranks showed that there were no significant differences between control samples and all the treated samples. There were also no significant differences between different treatments. An increase in cleaning duration did not produce a significant increase in the electrolyte leakage of the samples.

Discussion

Potential of UAS in improving microbiological safety and shelf-life extension

For a 40-second clean, samples cleaned with UAS were shown to have a significantly lower microbial load than samples cleaned with non-UAS on day 0. However, on day 6 post cleaning, there was no statistical difference between these sample groups. Many foodborne pathogens are known to enter the VBNC state during food processing stages which involves exposure to high temperature and an over-pressure, chemical disinfectants as well low storage temperature (Dinu and Bach 2011, Zhao et al. 2017, Highmore et al. 2018). Therefore, there

363 is a possibility that exposure to UAS might induce the bacteria to enter a VBNC state on day
364 0. Resuscitation of VBNC bacteria can be caused by influences such as the removal of the
365 inducing factors, changes in temperature, and the presence of chemical and biological stimuli
366 (Li et al. 2014, Zhao et al. 2017). After being stored at 4 ± 1^{o}C for 6 days, the VBNC bacteria
367 might regain culturability, and hence yielding the results as shown in Fig. 6. Previous studies
368 have shown that exposure to ultrasound can induce a VBNC state in *Legionella pneumophila*
369 (Declerck et al. 2010), and in *Salmonella typhimurium* when applied in combination with heat
370 (Liao et al. 2018). However, up to present, induction of the VBNC state in foodborne pathogens
371 caused by exposure to ultrasound without heat has not been investigated extensively. In future
372 studies relating to the removal of foodborne pathogens using ultrasonic cleaning technologies,
373 it would be worthwhile to compare detection methods such as fluorescence microscopy
374 (Highmore et al. 2018) or flow cytometry (Afari and Hung 2018) against culture-based methods
375 to validate the induction of VBNC state.

376 For a 2-minute clean, the effect of cleaning with UAS was more apparent on day 6 post
377 cleaning as the samples cleaned with UAS had a significantly lower microbial load than
378 samples cleaned without. Cleaning with UAS yielded a more promising result than non-UAS
379 as the microbubbles undergoing non-inertial cavitation could reach crevices in the leaf
380 surfaces to remove microbiological contaminants more thoroughly. This highlights the
381 potential of UAS in extending shelf-life. Here, the possibility of a 2-minute exposure to UAS
382 inducing bacteria to enter the VBNC state is not eliminated. As compared to a 40-second
383 exposure, a 2-minute exposure allows sufficient time for the UAS to disaggregate the biofilms
384 present on the leaf surface effectively and to detach the microbes from the surface. In this
385 case, the induction of bacteria into VBNC state does not affect the shelf-life test results as
386 much as that of a 40-second clean.

Effect of UAS on leaf quality

When cleaning with UAS, microbiological contaminants are detached from the leaf surfaces through the scrubbing action of bubbles undergoing non-inertial cavitation and the shear force generated in the surrounding liquid (Leighton, 2014; Maksimov and Leighton, 2018). The whole cleaning process does not involve the inertial collapse of bubbles, therefore minimising the risk of creating surface damage on the leaves. However, microscopic inspection at x400 magnification demonstrated that exposure to both UAS and non-UAS can lead to slight abrasions on the leaf surface, but the overall structure of the epidermal cells remained undamaged. The ΔZ measurements shown in Fig. 8 indicated that the degree of surface damage from these slight abrasions were not significantly different from the damage in the normal control, and significantly less than the damage induced in a control by normal handling with tweezers'. Electrolyte leakage measurements (Fig. 9) were used as an additional parameter to measure the effects of UAS cleaning on the leaf quality. The electrolyte leakage rates of samples treated with UAS for 2 minutes on day 0 were significantly higher than non-UAS (2 minutes) , but on day 6 post cleaning, the electrolyte leakage rates of all sample groups indicated that the physical quality of the leaves treated with different cleaning methods and durations were not significantly different from each other. This indicates that UAS did not cause significant surface abrasion that can negatively affect the overall leaf quality over time. Therefore, a cleaning duration of 2 minutes using UAS is deemed acceptable. However, for industrial applications, it would be beneficial to reduce the cleaning duration to reduce water consumption and at the same time achieve higher throughput efficiency.

Evaluation of cleaning efficacy

Interpreting laboratory testing results for food samples are often challenging as microbes are usually not homogenously distributed (Health Protection Agency, 2009). The initial microbial load of leaf samples used in this study represents the microbes naturally existing on the leaf phylloplane and may vary greatly between samples. Since salad leaves are highly perishable

and microbiological examination could not be performed on the same sample before and after cleaning, the cleaning efficacy was determined by comparing the average microbial load on samples which were after cleaning against uncleaned control samples.

As compared to whole leaf cleaning, leaf disc cleaning provided a more precise laboratory scale evaluation of the effects of UAS cleaning as the exposure conditions, i.e. contact time index, leaf disc cleaning remained constant between samples since the leaf discs have a controlled surface area between samples. However, it is not feasible to apply such a high contact time index for larger scale cleaning. Therefore, in the whole leaf cleaning experiment, a much lower contact time index was applied. As compared to leaf disc samples, whole leaf samples had variable surface areas between samples, but in overall, whole leaf samples are more representative to commercial cleaning of RTE salad leaves.

At present, nutrient agar was used in the cell culture method instead of selective agar as the main goal was to investigate if UAS can remove microbiological contaminants more effectively than non-UAS. TVC provides information on the effectiveness of a treatment but does not provide sufficient information on how or why some treatments are more effective than the others. Molecular detection of specific pathogenic bacterial species would be necessary in future experiments to carry out an in-depth investigation of the efficacy of UAS in improving the microbiological safety of salad leaves.

UAS devices for larger scale application in the RTE salad industry

Ultrasound has gained popularity in the food processing industry as it is an economically viable green technology, which is capable of reducing processing time and coping with higher throughput (Chemat et al. 2011, Jose et al. 2014). Other advantages of ultrasonic technologies also include the simplicity, scalability and energy efficiency of the system (Jose et al. 2014). In the Zhou et al. (2012) study, a key challenge for maintaining the cleaning efficacy of a commercial scale continuous flow ultrasonic cleaning system for fresh produce was the

attenuation of sound field when fresh produce was added into the cleaning system. Unlike immersive ultrasonic cleaning technologies, the UAS device used in this study does not require objects to be immersed in a liquid medium (Leighton 2015), therefore such a problem would not exist in a UAS system. Moreover, without immersion, the risk of cross contamination could be reduced.

A major limitation of the UAS device used in this study is that it can only clean one sample at a time, although it demonstrates potential in improving the microbiological safety and shelf-life of RTE salads. A future solution to this would be the adaptation of the cleaning mechanism of UAS in the development of a novel ultrasonic cleaning technology that can cope with a much higher throughput. To increase the possibility of a novel cleaning technology to be adopted in the RTE food industry, it would be necessary to prove that the proposed technology is capable of achieving effective removal of pathogenic foodborne pathogens such as *E. coli O157:H7*, *Salmonella enterica* and *Listeria monocytogenes* without affecting the product quality. This indicates that further evaluation and in-depth research related to the cleaning efficacy and the adaptability of UAS cleaning mechanism in an industrial scale is necessary. In addition, when designing UAS cleaning systems for the fresh produce industry, it is also crucial to ensure that the energy consumption is kept minimal so that the additional costs incurred will not be a factor that hinders the implementation of the technology.

Conclusions

The efficacy of UAS in cleaning spinach leaves was evaluated. The leaf disc cleaning results showed that at a cleaning duration of 40 seconds, UAS cleaned significantly better than non-UAS, whereas there was no significant difference between both methods at a cleaning duration of 20 seconds (Fig. 5). For further evaluation, the cleaning experiment was repeated with whole spinach leaves from different salad fields. Samples cleaned with UAS for 2 minutes had a significantly higher microbiological quality than samples cleaned with non-UAS on day 6 post cleaning (Fig. 6). The cleaning durations used in both leaf disc cleaning and whole leaf

cleaning studies are limited to showing the effects of increased treatment times. Further controlled studies are required to investigate the minimum cleaning duration needed to produce significant cleaning effects. Microscopic inspection and electrolyte leakage measurements also showed that an exposure to UAS for 2 minutes did not produce significant damage on spinach leaf surface that can adversely affect the overall leaf quality or product shelf-life. These results highlight the potential of UAS in improving the microbiological safety to reduce the incidence of food poisoning for fresh produce (CDC 2018, 2020). Following industry advice, the microbiological load was used as a predictor of the shelf-life of spinach leaves. There were indications of the potential for UAS to increase this, an important goal given that, worldwide, one-third of the food produced for human consumption is either spoiled or wasted (Food and Agriculture Organization of the United Nations, 2011). Limitations of food shelf-life lead to food insecurity, migration, and malnutrition, and increases in fossil fuel usage to meet transportation and refrigeration demands (Hammond et al. 2015), all of which impact upon public health. Further evaluation on the ability of UAS to enhance the traditional cleaning methods of detaching certain species of foodborne pathogens and spoilage microorganisms, as well as investigation of whether UAS causes the induction of foodborne pathogens into the VBNC state, are necessary in the development of this novel ultrasonic cleaning technology for industrial implementation.

Acknowledgements

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Conflict of Interest Statement

The principal investigator (T.G.L.) is the inventor of the UAS device used in the experimental work, and is a Director and Inventor-in-Chief of the company (Sloan Water Technology, Ltd.) which owns the rights to the technology. However, he has taken no salary or other remuneration for these roles. Another author (C.N.D.) is currently an employee of that company.

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Figure Captions List

Fig. 1 In (a), excited by the 132 kHz field to a pulsation motion that is too small to discern on this scale, bubbles (i) and (ii) exhibit surface waves, but bubble (iii) is too large to undergo anything but the zeroth order spherical harmonic perturbation (as predicted by theory; Maksimov and Leighton, 2001) and would require a higher amplitude sound field to host surface waves. The sound field is switched off 125 μs later in panel (b), and the consequent cessation of surface waves shows a clear difference (i.e. the loss of the ripples in panel (a), that Faraday (1931) characterised as criss-crossing 'crispations' when he observed them on macroscopic flat liquid surfaces). These are particularly clear where the nearly-parallel bubble walls near the centre of the image allow the light to shine through it. Bubble (ii) and (iii) and held against a thin wire by Bjerknes forces; and bubble (i) is similarly attracted to a crack in a glass side held in the focal plane.

Fig. 2 (a) Schematic diagram of the experimental setup (adapted from Malakoutikhah et al. 2020); (b) Schematic of the leaf surface. The stomata are controlled by the guard cells to allow oxygen in, and carbon dioxide out, but closed periodically to prevent excessive water loss; (c) Microscopic images showing the surface topography of the (i) adaxial and (ii) abaxial surfaces of a control (uncleaned) spinach leaf sample, scale bar = 100 μm. The positions of the stomata are indicated using white arrows.

Fig. 3 The variation in the acoustic pressure amplitude recorded using a Reson TC4038 hydrophone placed at a position where the acoustic centre of the hydrophone was at 5 mm below the nozzle of the UAS device over a period of 20 minutes. The mean power of the electrical supply for transducer featuring signal generation and power amplification. Error bars represent the standard deviation of 4 repeats.

Fig. 4 Illustration showing (a) the positions at which the microscopic images were acquired on each leaf disc. The distance between both the horizontal and vertical adjacent spots are 2 mm.

(b) The distance between the top (Z_1) and bottom (Z_0) focal position, ΔZ in micrographs of (i) an uneven surface; (ii) a wounded region at magnification levels (iii) x100 and (iv) x400 respectively.

Fig. 5 Mean microbial load remaining on leaf discs cleaned with different methods and durations. Error bars represent the standard error of mean of 15 samples. Statistical difference between (i) control and treated samples; (ii) treatment groups were shown in dotted and solid lines respectively, where non-significance (ns) = $P > 0.05$, and ** = $P \leq 0.01$, *** = $P \leq 0.001$.

Fig. 6 Mean microbial load remaining on whole leaf samples on day 0 and day 6 post cleaning. Error bars represent the standard error of mean of 6 repeats. Statistical difference between (i) control and treated samples; (ii) treatment groups were shown in dotted and solid lines respectively, where non-significance (ns) = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$ and *** = $P \leqslant 0.001$.

Fig. 7 Representative microscopic images showing the adaxial and abaxial surfaces of damaged and undamaged control spinach leaf samples as well as samples treated with and without UAS at both durations. Scale bar = 100 µm. Surface abrasion caused by each cleaning treatment is indicated using red arrows. The distance between the top and bottom focal position, ΔZ of each micrograph is as labelled. The damaged control micrograph was acquired from leaf disc with manually created wounds which were visible at a macroscopic scale.

Fig. 8 Mean distance between the top (Z_1) and bottom (Z_0) focal position, ΔZ at x100 magnification and x400 magnification. Adaxial and abaxial surfaces are denoted as AD and AB respectively. Error bars represent the standard deviation between 9 micrographs. Statistical difference between both control (damaged and undamaged) samples and treated samples are shown in solid lines, where non-significance (ns) = $P > 0.05$, * = $P \leqslant 0.05$, ** = $P \leqslant 0.01$ and *** = $P \leqslant 0.001$.

721 **Fig. 9** Mean electrolyte leakage of whole leaf samples treated with and without UAS for 40 s
722 and 2 mins. Error bars representing the standard error of mean of 10 samples. Statistical
723 difference between (i) control and treated samples; (ii) treatment groups were shown in dotted
724 and solid lines respectively, where non-significance (ns) = $P > 0.05$, * = $P \leq 0.05$.

Table

Table 1 The contact time indexes of both sample types treated with different cleaning duration.

Sample type	Area of sample (cm^2)	Cleaning duration each side, T (s)	Contact time index
Leaf disc	0.6 *	10	26.12
	0.6 *	20	52.24
Whole leaf	28 ± 4	20	1.12 ± 0.16
	28 ± 4	60	3.36 ± 0.48

* Corrected to one decimal place

Figure 1

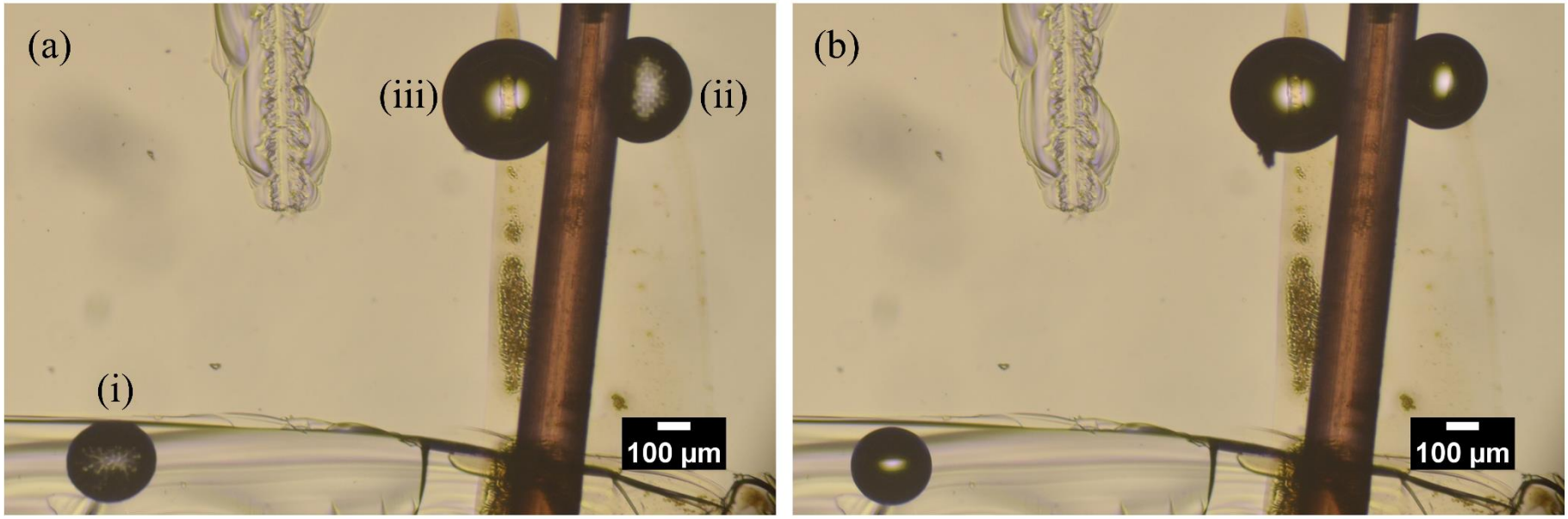

Figure 2

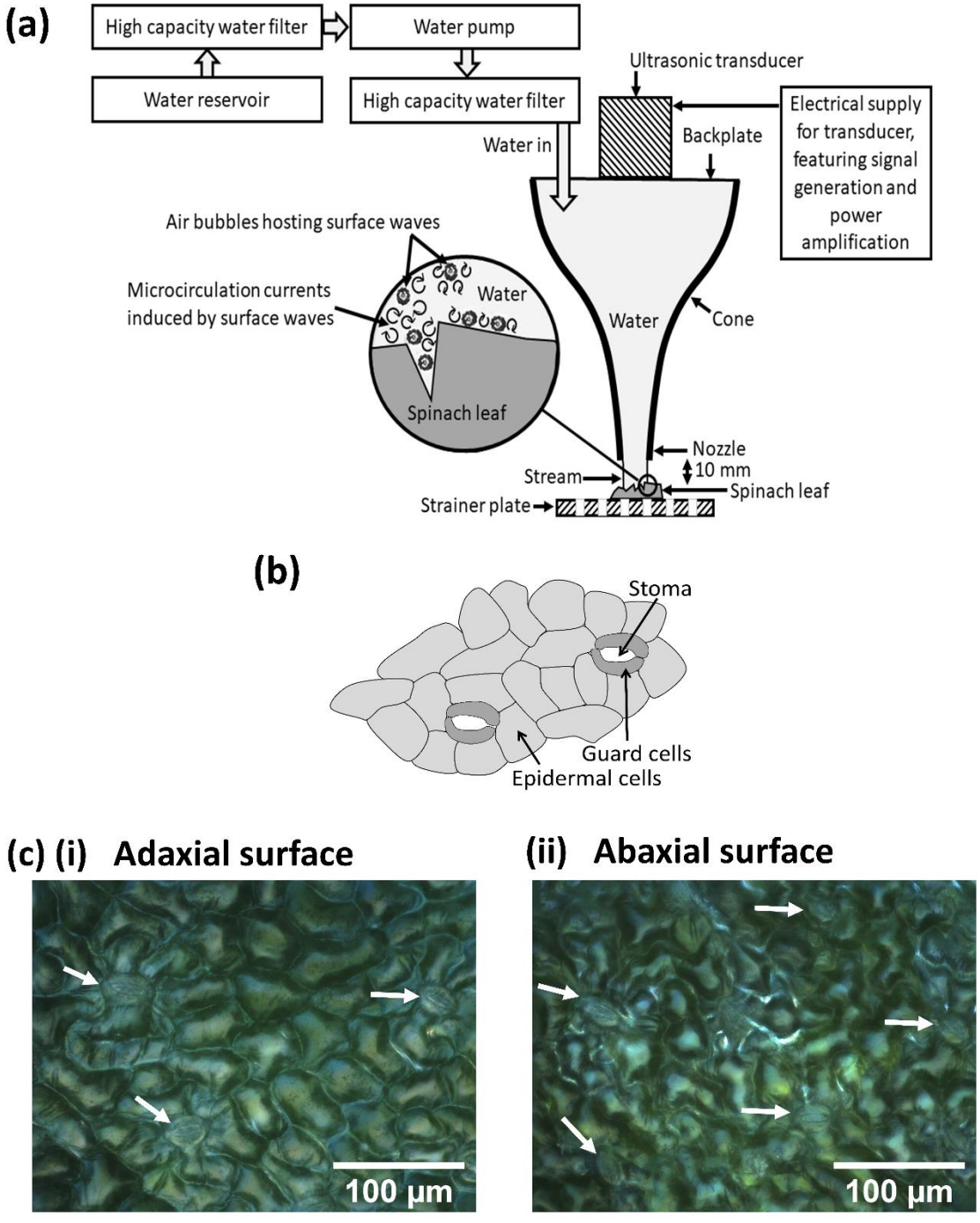
(a)
High capacity water filter
Water pump
Water reservoir
High capacity water filter
Ultrasonic transducer
Electrical supply for transducer, featuring signal generation and power amplification
Backplate
Water in
Air bubbles hosting surface waves
Microcirculation currents induced by surface waves
Water
Water
Cone
Spinach leaf
Nozzle
10 mm
Stream
Spinach leaf
Strainer plate
(b)
Stoma
Guard cells
Epidermal cells
(c) (i) Adaxial surface
100 µm
(ii) Abaxial surface
100 µm

Figure 3

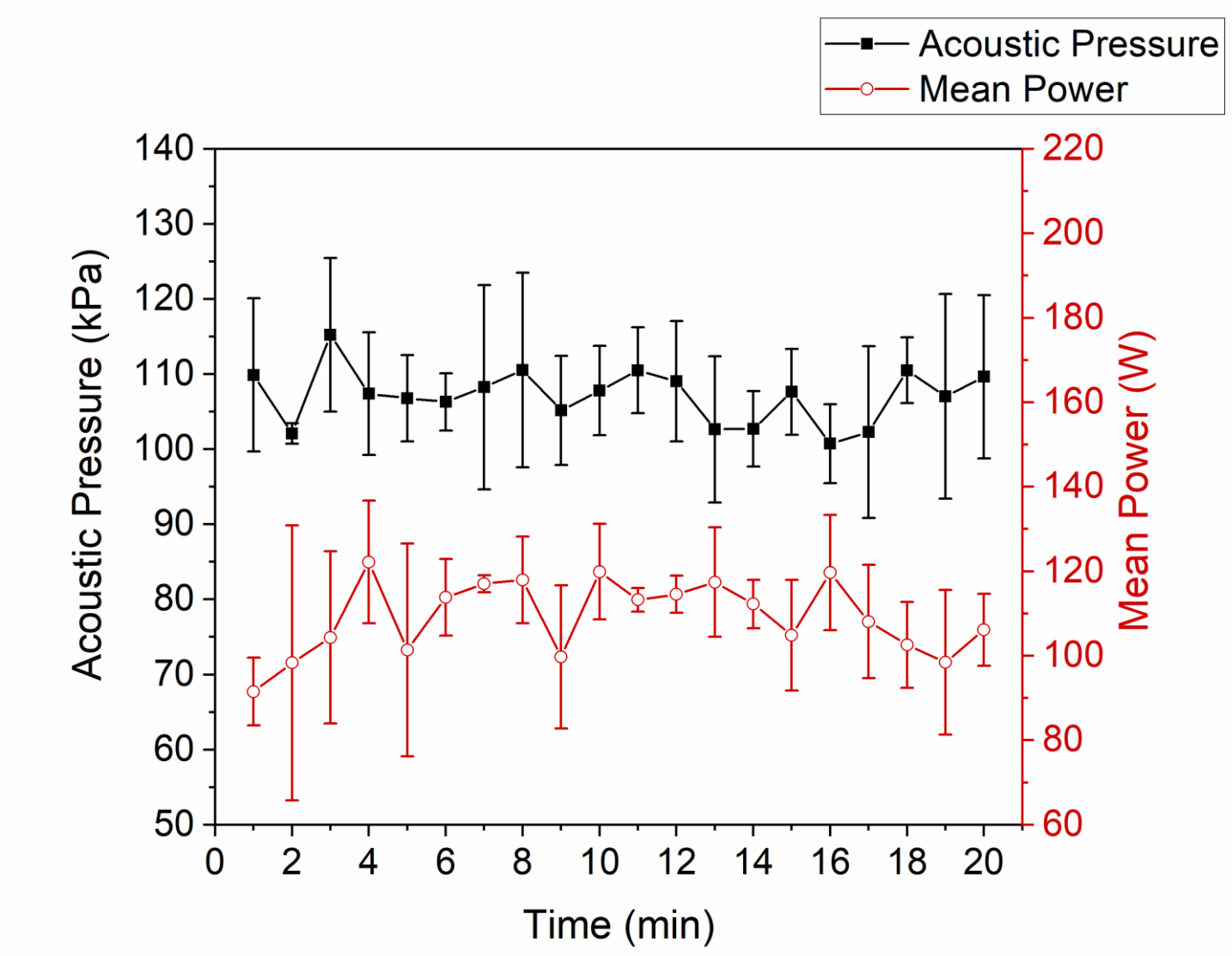
Acoustic Pressure
Mean Power
Acoustic Pressure (kPa)
Mean Power (W)
Time (min)
140
130
120
110
100
90
80
70
60
50
220
200
180
160
140
120
100
80
60
0
2
4
6
8
10
12
14
16
18
20

Figure 4

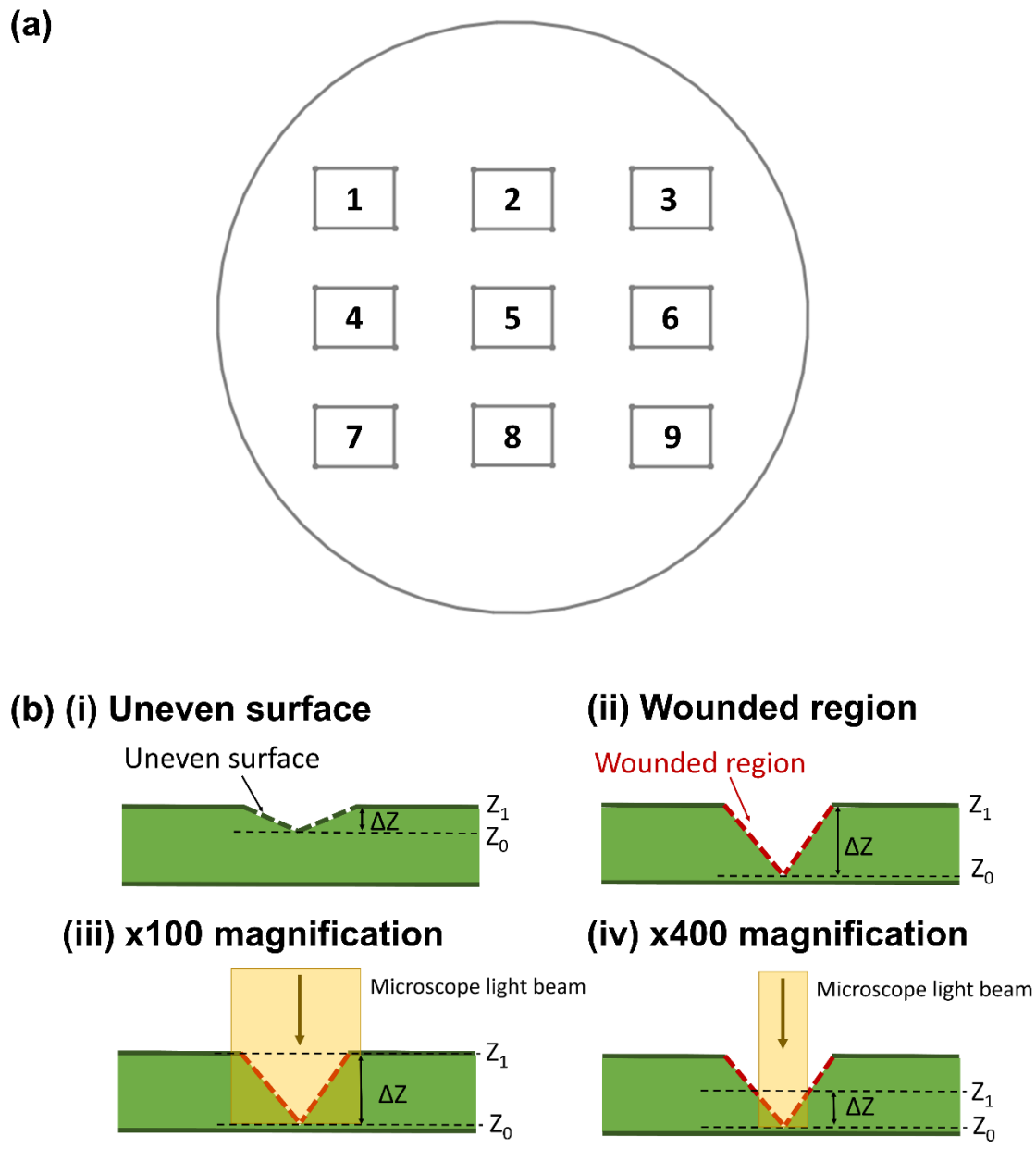
(a)
1
2
3
4
5
6
7
8
9
(b) (i) Uneven surface
Uneven surface
ΔZ
Z1
Z0
(ii) Wounded region
Wounded region
ΔZ
Z1
Z0
(iii) x100 magnification
Microscope light beam
ΔZ
Z1
Z0
(iv) x400 magnification
Microscope light beam
ΔZ
Z1
Z0

Figure 5

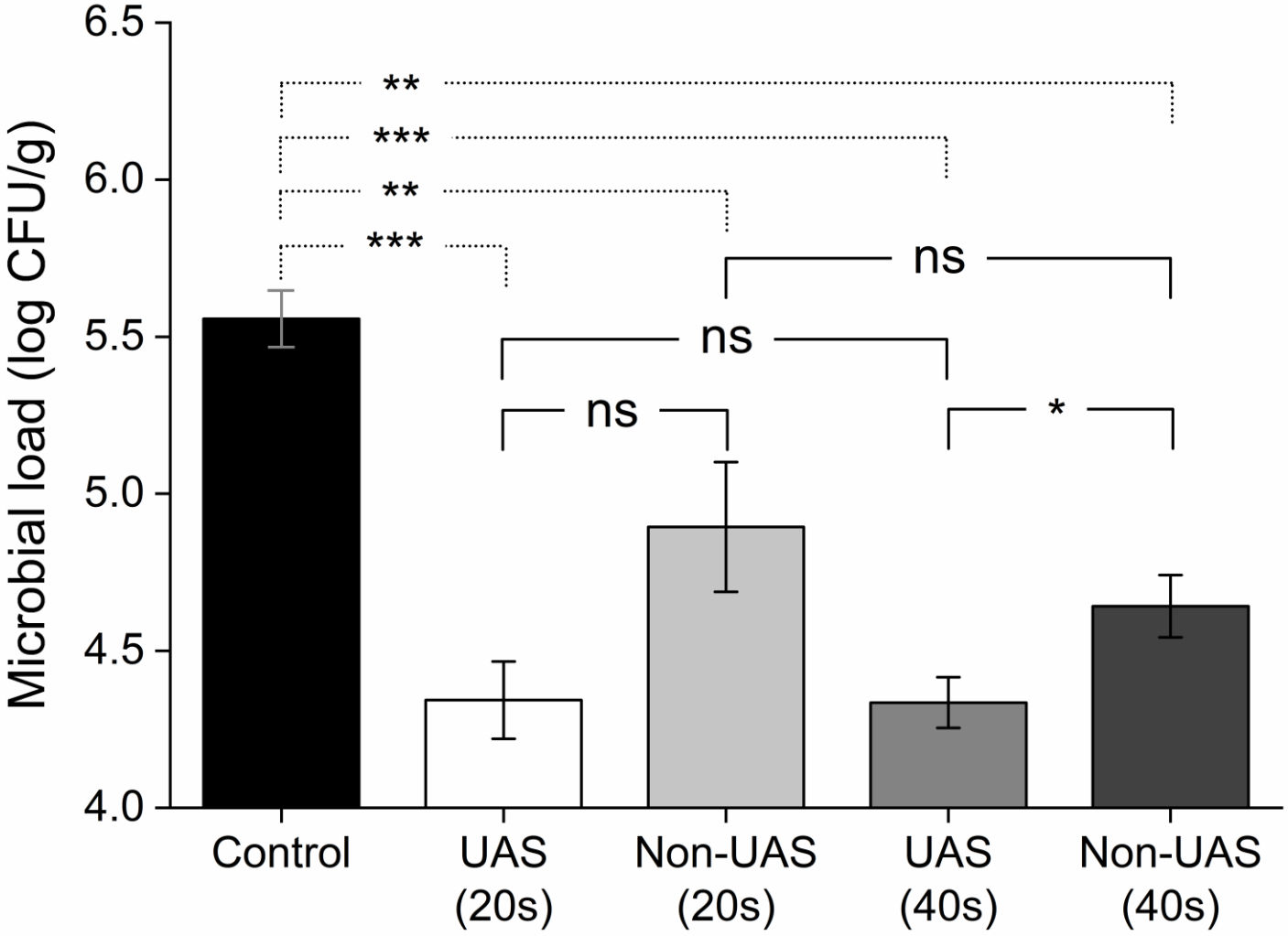
Microbial load (log CFU/g)
6.5
6.0
5.5
5.0
4.5
4.0
**
***
**
***
ns
ns
ns
*
Control
UAS (20s)
Non-UAS (20s)
UAS (40s)
Non-UAS (40s)

Figure 6

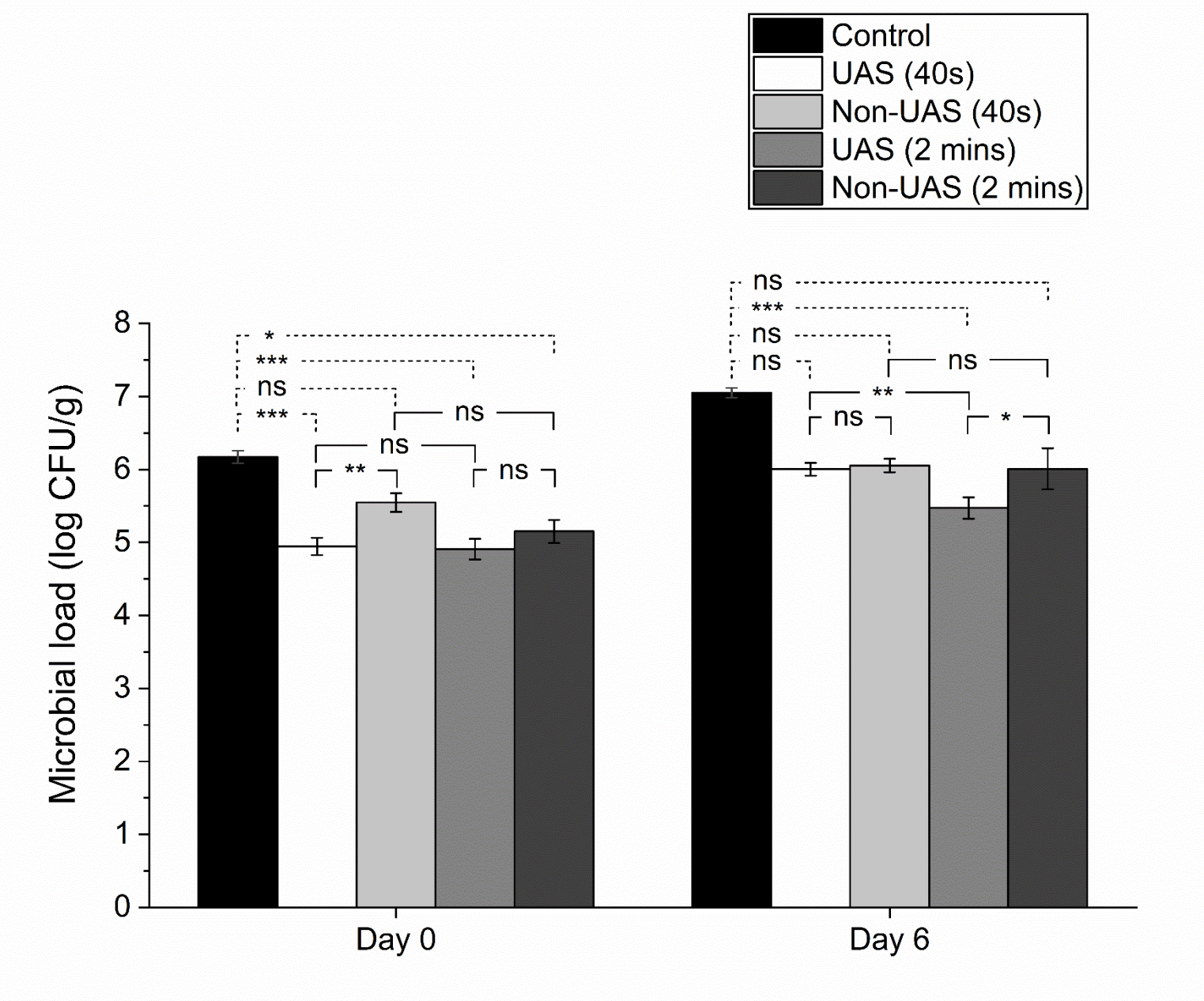
Control
UAS (40s)
Non-UAS (40s)
UAS (2 mins)
Non-UAS (2 mins)
Microbial load (log CFU/g)
0
1
2
3
4
5
6
7
8
*
***
ns
***
ns
ns
**
ns
ns
***
ns
ns
**
ns
*
Day 0
Day 6

Figure 7

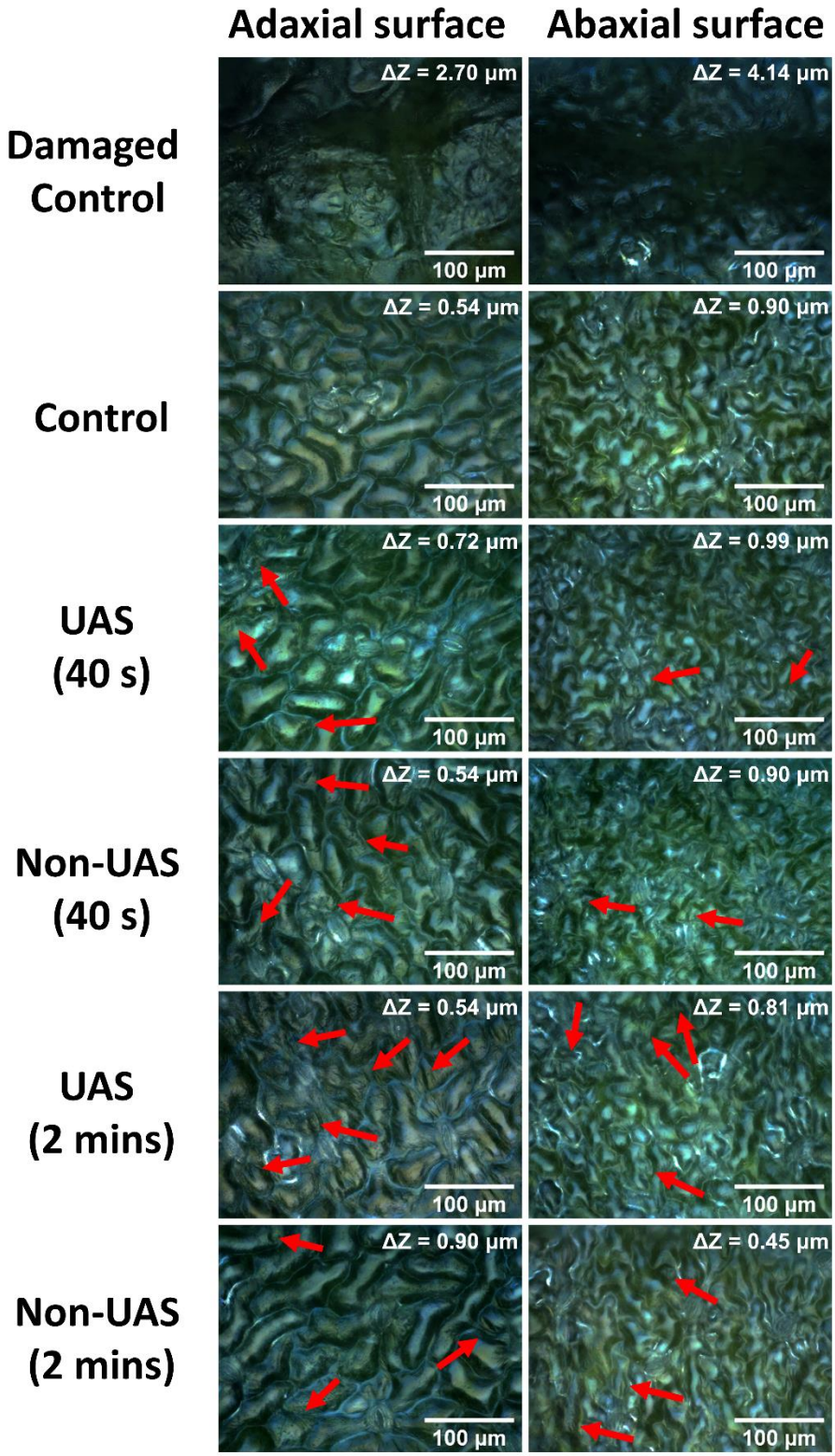
Adaxial surface
Abaxial surface
Damaged Control
ΔZ = 2.70 μm
ΔZ = 4.14 μm
Control
ΔZ = 0.54 μm
ΔZ = 0.90 μm
UAS (40 s)
ΔZ = 0.72 μm
ΔZ = 0.99 μm
Non-UAS (40 s)
ΔZ = 0.54 μm
ΔZ = 0.90 μm
UAS (2 mins)
ΔZ = 0.54 μm
ΔZ = 0.81 μm
Non-UAS (2 mins)
ΔZ = 0.90 μm
ΔZ = 0.45 μm
100 μm

Figure 8

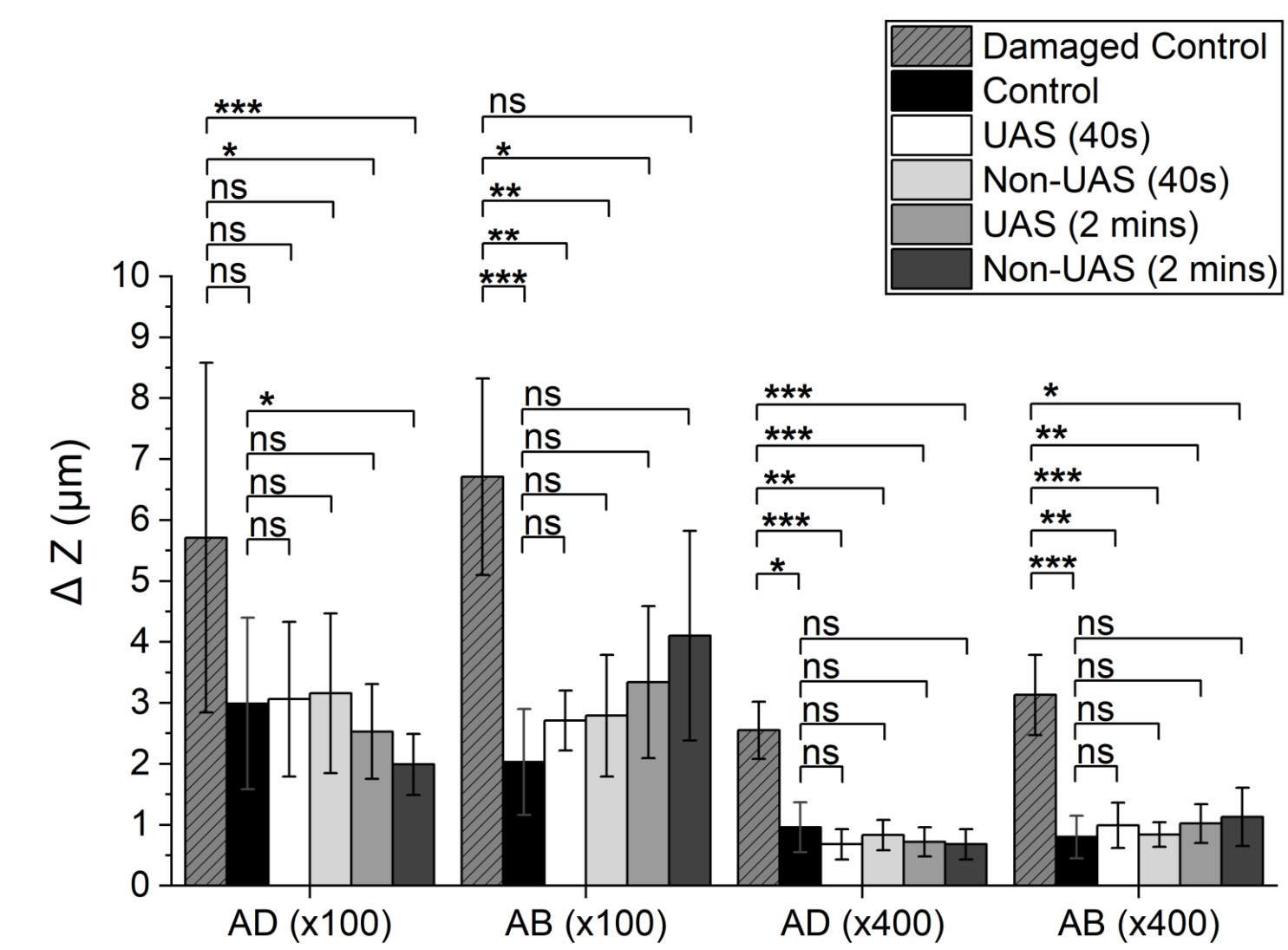
Damaged Control
Control
UAS (40s)
Non-UAS (40s)
UAS (2 mins)
Non-UAS (2 mins)
Δ Z (μm)
0
1
2
3
4
5
6
7
8
9
10
AD (x100)
AB (x100)
AD (x400)
AB (x400)
***
*
ns
ns
ns
ns
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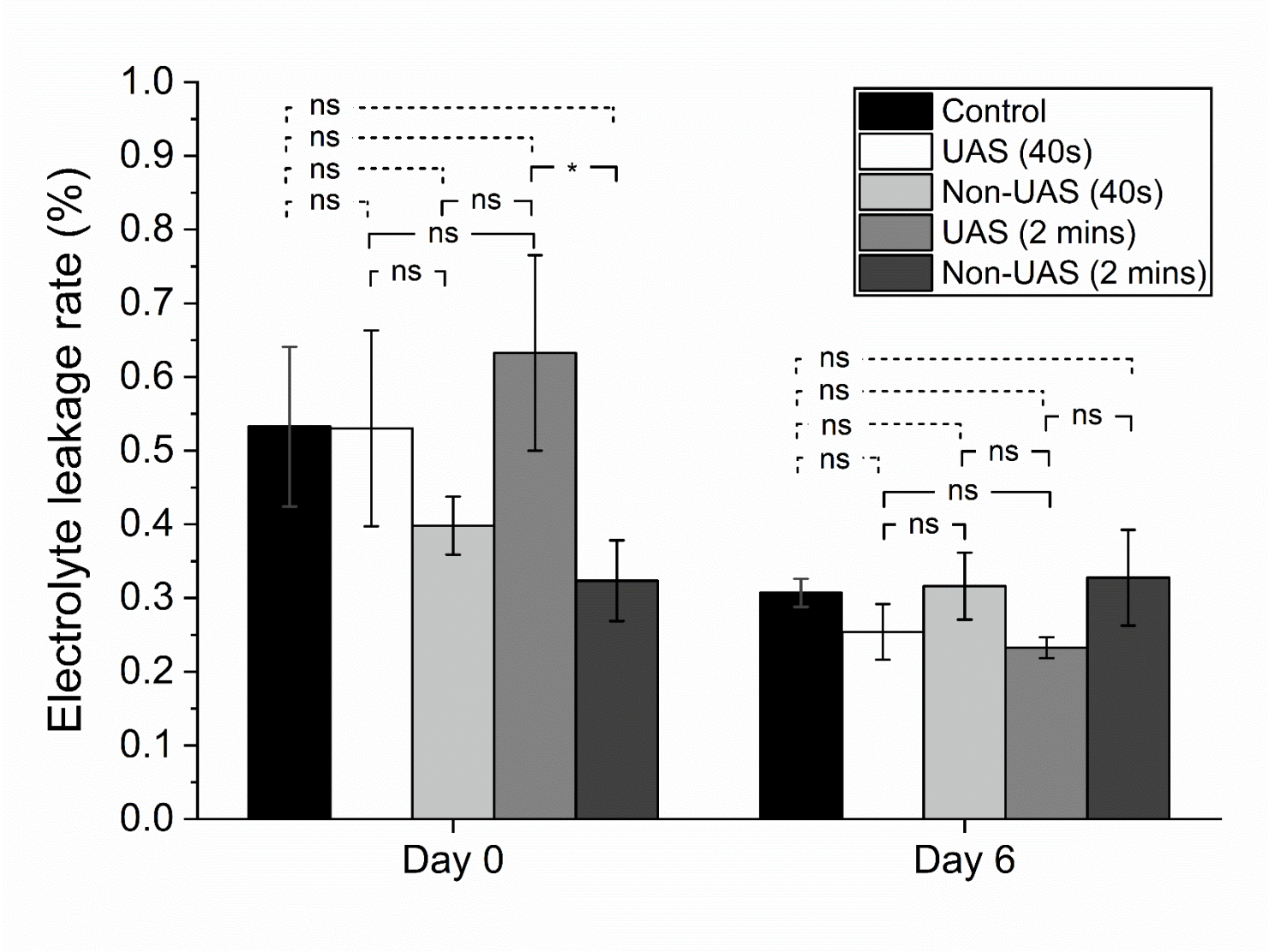
Figure 9


Electrolyte leakage rate (%)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Day 0
Day 6
Control
UAS (40s)
Non-UAS (40s)
UAS (2 mins)
Non-UAS (2 mins)
ns
*