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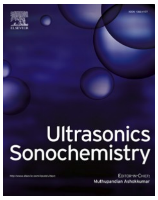

Improving livestock feed safety and infection prevention: Removal of bacterial contaminants from hay using cold water, bubbles and ultrasound

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ABSTRACT

The ingestion of contaminated hay is detrimental to livestock wellbeing. In this study, the feasibility of using an ultrasonically activated stream (UAS) to clean bacterial contamination from hay was investigated. Hay samples were stained with SYTO-9 nucleic acid stain for the *in-situ* visualization of microbes on the surface using an episcopic differential interference contrast microscope coupled with epi-fluorescence. The total microbial load per sample was calculated by measuring the mean percentage area of SYTO-9 positive staining. The cleaning efficacy was evaluated by comparing the total microbial coverage before and after cleaning. The cleaning performance between an UAS and a non UAS were compared and results have shown that an exposure of 60 s to an UAS demonstrated an 87.94 ± 2.22% removal of the bacterial contaminants, exceeding that of non UAS (21.85 ± 13.63% removal). UAS is capable of removing bacterial contaminants without the use of antimicrobial agents, therefore its cleaning mechanism can potentially prevent infection and reduce antimicrobial resistance. The cleaning mechanism of UAS can be adapted for the development of a new hay cleaning strategy for effective removal of bacterial contaminant to improve feed safety.

1. Introduction

With the increasing economic value of the equine sector [1], attention must be paid to the health of the horses involved. Studies have demonstrated that the etiopathogenesis of equine respiratory disease is associated with poor quality hay contaminated with dust and aero-allergens [2,3]. Apart from respirable dust, hay is also susceptible to bacterial and fungal contamination especially when kept in high moisture environments [4]. Two commonly practised cleaning methods for hay are soaking and steaming. Soaking is commonly used to reduce the amount of airborne respirable particles [5]. However, post-soak water is also likely to provide a medium for bacterial proliferation and recontamination of the hay surface [6]. On the other hand, steaming reduces the level of bacteria and mould present on hay [7]. However, it has a tendency to leave the mould in place on the hay surface after killing it [7]. A major disadvantage of hay steaming is its high power consumption. Here we present an alternative cleaning method that might overcome some of the limitations of these existing methods.

In contrast to steaming, ultrasonic cleaning is more environmentally friendly as heating of wash water is not required throughout the process [8,9]. Whilst cleaning baths have a proven efficacy for many applications [10,11] in the context of cleaning delicate materials they have drawbacks [9]. Such units rely on transient or inertial cavitation induced by high power ultrasound for their cleaning action [12,13]. The violent collapse of bubbles causes the cleaning, but this process can generate free radicals [14,15] and blast waves [16] and consequently may damage biological tissue and rupture cells [17,18]. In addition, these baths can only treat items small enough to fit within them [19]; whilst keeping the item being cleaned in a soup of contaminated material removed from them, which can cross-contaminate further objects used within the ultrasonic bath [9]. Notably for the concept of cleaning hay in bulk, cleaning baths rely on the propagation of ultrasound throughout the bath water, and the gas spaces in hay [20] will attenuate the sound field, reducing its ability to treat a batch [21].

An alternative ultrasonic cleaning is an Ultrasonically Activated Stream (UAS) [22,23]. The UAS system ensures that bubbles circulating

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in the water flow are excited on the surface of the target to be cleaned (here, hay) by an ultrasound field with an amplitude at a sufficient level to generate surface waves on the bubble, but not generate inertial cavitation [24]. This can be achieved in a number of ways, most conveniently by supplying fresh mains taps water into the acoustic cone that generates the ultrasound and from which the stream issues. However in repeated laboratory tests this leads to an unacceptable wastage of water, and the device shown in Fig. 1 uses a recirculating water supply (refilled each day with mains tap water). Run-off from the test sample is pumped back into the device, and contains a population of small bubbles that are suitable, when they reach the target, of hosting Faraday wave and other surface waves [25,26] which can be stimulated to grow under the correct conditions (Fig. 2) [27,28]. These surface waves on the walls of bubbles exert convection [29,30] and shear forces [14] in the surrounding liquid, producing a cleaning effect as the stream is projected onto the contaminated surface [24]. Furthermore, acoustic radiation forces on the bubbles [31] cause them to be attracted into the crevices [14,32,33]. The effectiveness of the UAS systems has been demonstrated [24,34] including the removal of ‘sooty’ particulate contaminants from equipment on railway locomotives [35]; biofilms associated with marine biofouling [36] and dental bacteria [37]; lubricant [38] and hazardous infectious biological contamination from surgical steel [39] and from bone being prepared as for transplant [40].

This paper investigates the efficiency of an ultrasonically-enhanced cold water rinse for use in cleaning hay for feeding. This is done by comparing the cleaning results to those of a normal water rinse and aims to justify whether it is worth the larger enterprise of comparing against steaming and soaking, and investigating how UAS might be incorporated into bulk processing apparatus.

2. Material and methods

2.1. Visualization of the microbiological conditions of hay samples

Each hay sample was inoculated with 10 μM SYTO-9 Green Fluorescent Nucleic Acid Stain (Thermofisher Scientific) in phosphate buffered saline (PBS) for 10 min at room temperature. The samples were wrapped in aluminium foil to prevent exposure to light throughout the staining process. Once stained, the samples were then rinsed with PBS followed by deionised water. For each sample, 10 randomly distributed fields of view were captured using the episcopic differential interference contrast (EDIC) microscope at a magnification value of 100 under the fluorescein isothiocyanate (FITC) filter. Images were acquired using ImagePro software (MediaCybernics) at an exposure value of 150 ms (ms) for grass-like samples and 50 ms for straw-like samples. After cleaning, a further 10 randomly distributed microscopic fields of view were taken for each sample.

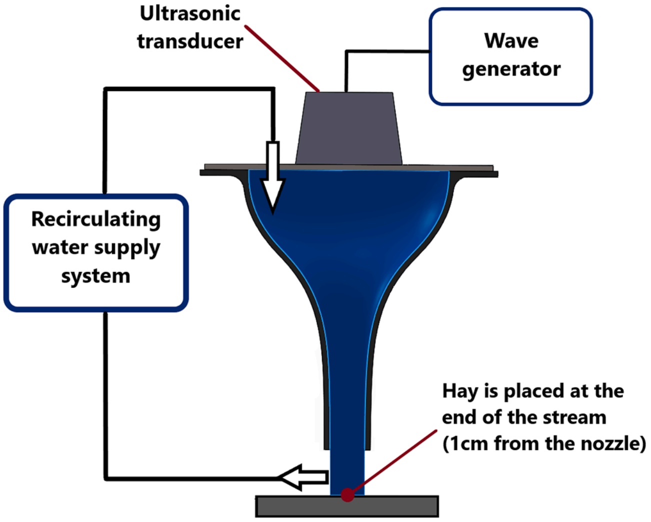

Fig. 1. (colour online) A schematic illustration of a UAS cleaning system.

2.2. Cleaning with cold water and UAS

The apparatus is shown in Fig. 1. Before impacting the hay, water from the UK Mains supply (without any additional treatment or additives) was poured into a recirculating water supply system, then, passed through the UAS device, either with the ultrasound activated (to correspond to normal UAS operation) or with it off, which corresponds to cleaning with normal mains supply cold water. The UAS was generated using a StarStream Mark 1 (Mk 1) device [22]. The experimental setup comprises the StarStream Mk 1 device and a water recirculating system. The recirculating system was used to demonstrate cleaning using a water-conservation principle. The water flow rate was set at 2.00 ± 0.04 L per minute throughout all experimentation. For UAS cleaning, a wave generator (custom built under licence by Ultrawave Ltd) was connected to the ultrasonic transducer of the device. The ultrasonic frequency was 132 kHz. The device consumed 100 W of electrical power [8], and the wave generator was designed to generate ultrasonic waves with acoustic pressure amplitude that is sufficiently high to overcome the threshold amplitude for non-inertial cavitation. At 132 kHz, the Blake threshold (the minimum condition that must be exceeded to generate inertial cavitation) ranges smoothly from 140 – 200 kPa (zero to peak) between over a range that exceeds the maximum and minimum bubble sizes that could be present (1–1000 μm radius). Hydrophone measurement of acoustic pressure amplitude of the UAS at the target was 120 kPa zero-to-peak, although such data must be used in the knowledge that if the measurement conditions (here, a hydrophone in a stream) do not match the calibration conditions (a hydrophone in an effectively infinite body of liquid) in terms of the acoustic properties of the environment (as here), then the calibration supplied with the device cannot be assumed to be accurate, and indeed no calibration can be used unless a certified national measurement facility sets up calibrations for these stream conditions (of which none exists) [32,40]. Therefore confirmation, to complement these hydrophone measurements, that this device does not produce inertial cavitation on the target, was obtained through observation that it produced no sonoluminescence, and by confirming by microscopic analysis that fragile targets suffered no damage.

One at a time, the hay samples were held at both ends using forceps and moved through the water stream in a to-and-fro motion repeatedly for 1 min to ensure the entire hay surface was being cleaned. The distance between the hay sample and nozzle was kept at 1 cm. For cold water cleaning, the hay was cleaned following the same procedure but without ultrasonic activation of the stream.

2.3. Image and data analysis

The microscopic images were analysed using ImageJ (National Institutes of Health) to measure the percentage area of the SYTO-9 positive green fluorescent microbes present on the samples. The percentage remaining before and after cleaning was used to estimate the cleaning performance of the tested methods. The calculation for the percentage remaining achieved by each of the cleaning methods can be found in Table 1. The standard error of mean (SEM) was calculated for each set of data. The data was analysed using one-way analysis of variance (ANOVA) followed by a post hoc Tukey’s Multi Comparison Test for the evaluation of data significance.

3. Results

3.1. Cleaning performances of UAS and cold water

The experimental results indicate that UAS is more effective in

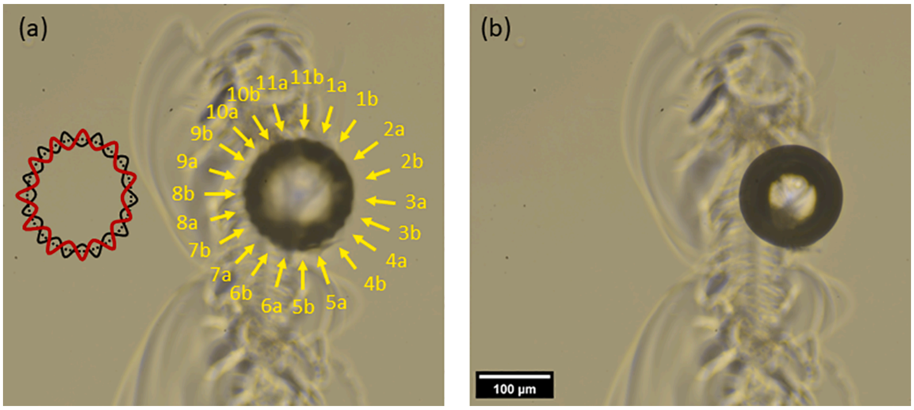

Fig. 2. (colour online) A bubble of radius 40 μm in the 132 kHz field of the device, as seen through a glass plate inserted between the water and the camera. A scratch, running vertically though the image, is visible in the glass, and Bjerknes forces have attracted the bubble to it. Panel (a) is taken just before the sound field is turned off (at which point panel (b) is taken). The exposure is 1/4000 s, which integrates over nearly 22 cycles of the Faraday wave motion, such that at any one time, half the peaks numbered in (a) (1a, 2a, 3a etc.; shown schematically by the thin black line to the left of the imaged bubble in panel (a)) are peaks and the other half (1b, 2b, 3b etc.; shown schematically by the thick red line to the left of the imaged bubble in panel (a)) are troughs. Half a cycle of the Faraday wave later, they swap over, and over the nearly 22 cycles of the frame exposure, both sets show as peaks in the bubble wall imaged. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
The calculation of the percentage remaining on the samples after cleaning. The sample mean is calculated by averaging the percentage remaining on the sample based on the 10 microscopic images (n = 10) for each sample. All values were corrected to four decimal places.

Cleaning Method	Hay type	Sample	Sample Mean (%) (n = 10)	Group Mean (%)	Percentage Remaining (%)
UAS	Straw-like	UAS_SL_1	15.3401	15.7856	12.0594
		UAS_SL_2	14.9350		
		UAS_SL_3	13.8107		
		UAS_SL_4	15.3055		
		UAS_SL_5	19.5366		
	Grass Like	UAS_GL_1	1.0309	8.3331	
		UAS_GL_2	8.1997		
		UAS_GL_3	4.6228		
		UAS_GL_4	22.7893		
		UAS_GL_5	5.0230		
Water Wash	Straw-like	WW_SL_1	34.8138	63.7663	78.1543
		WW_SL_2	29.3244		
		WW_SL_3	120.0773		
		WW_SL_4	17.0706		
		WW_SL_5	117.5453		
	Grass Like	WW_GL_1	108.5216	92.5424	
		WW_GL_2	54.4457		
		WW_GL_3	106.9135		
		WW_GL_4	131.3740		
		WW_GL_5	61.4570		

removing bacteria from the hay surface as it achieved a percentage reduction of 87.94 ± 2.22% whereas non UAS achieved 21.85 ± 13.63%. A Tukey's Multiple Comparisons Test was used to compare these results, which produced an adjusted P value of <0.0001 for the comparisons between UAS and either the water wash or uncleaned control samples, indicating a significance difference in the level of bacteria present through cleaning with UAS. Using the same test, a lack of significance ($P = 0.1478$) between the uncleaned and non UAS results was seen indicating that cold water wash does not significantly reduce the microbial load of hay when used alone. Furthermore, an increase in microbial load was observed in a few samples cleaned with non UAS (See Table 1).

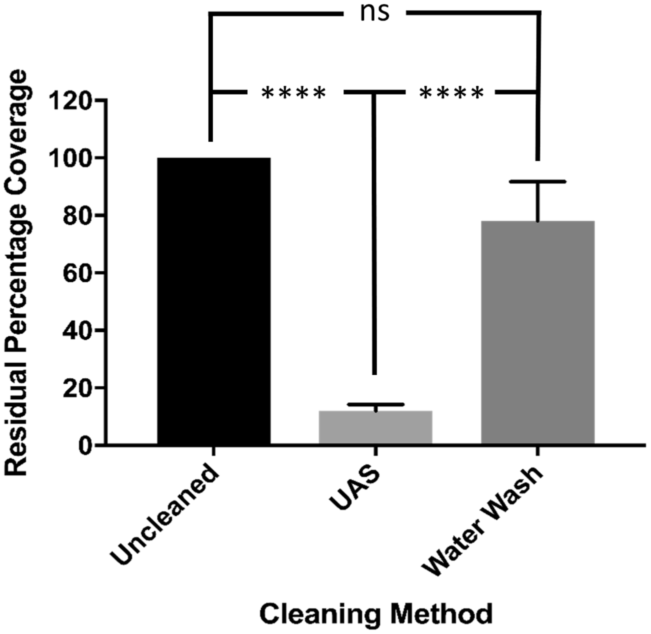

Fig. 3. Analysis of the EDIC microscopy images (n = 10 samples, each with 10 random fields of view) showing the percentage remaining on the sample after being cleaned using UAS and water for all the samples regardless of their topographical features. The uncleaned results are set to 100% by default and the error bars represent the SEM in each case. Significance results represent Tukey's Multiple Comparisons Test, where non significance (ns) = $P > 0.05$, * = $P \leq 0.05$, ** = ≤ 0.01, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

3.2. *Percentage removal from different topographical features*

Following on from the results shown in Fig. 3, the hay samples and thus their cleaning results can be divided into two groups based on their topographical properties. These groups have been labelled straw-like and grass-like, the former indicating a firm cylindrical structure, and the latter indicating a less-brittle flatter structure, representative examples of which are shown in Fig. 4. As shown in Fig. 5, for straw-like samples, the percentage reduction of area covered by bacteria after cleaning with UAS was 84.21 ± 0.98%, while water wash was able to remove 36.23 ± 22.66% of the contaminants. A Tukey's Multiple Comparisons Test was again used to test for the significance of these results, with cleaning using UAS producing a significant effect on the level of microbial coverage with an adjusted P value of 0.0008 compared to the control, while cleaning with just a water failed to produce a significant effect with an adjusted P value of 0.2607 compared to the control. For grass-like samples, UAS achieved a reduction of 91.67 ± 3.79% whereas water wash removed 7.46 ± 14.81%. Using the same significance test the same result as with straw-like samples was seen, with UAS producing a significant effect with an adjusted P value of

Fig. 4. (colour online) Representative examples of the two topographically distinct groups of hay used in these experiments, where: **1** indicates the “grass-like” hay with a less-brittle flatter structure; **2** indicates “straw-like” hay with a firm cylindrical structure.

0.0003 and water producing a non-significant effect with an adjusted P value of 0.9922, both when compared to the control samples.

Therefore, as with the combined result, unlike UAS cleaning, a cold water wash showed no significant effect on the microbial load of either topographical group. The grass-type hay also displayed a significant difference between the UAS result and the water wash result, which is lost when doing this same comparison for the straw-like hay. It can be observed from Fig. 5 that there is much more variation between the water wash results when comparing straw-like and grass-like than when comparing the UAS results for each group, thus the loss of significance might result from effects seen with the water wash rather than with the UAS.

4. Discussion

With the value of the equine sector on the rise and hay being the primary source of the contaminants responsible for equine respiratory diseases, an effective method of reducing the contaminants on hay is essential [41,42]. Existing methods for cleaning hay have included soaking and steaming, however both of these previously established cleaning methods have drawbacks, including lengthy cleaning times and increasing the moisture level of the hay, which in turn increases the chance of re-contamination if the hay is not consumed soon after cleaning [6]. Here we have looked at the possibility of using new ultrasonic technology to clean hay. This technology uses an Ultrasonically Activated Stream (UAS) device, which projects ultrasound down a stream of water, stimulating Faraday waves and higher order surface waves on the surface of the bubbles on the hay. This is turn sets up liquid microstreaming currents in the liquid next to the hay [24], removing contaminant (Fig. 6). Technology that cleans without chemicals (particularly chemicals that would then be present in the water run-off to return to the wastewater supplies, rivers, and field etc.) is particularly useful for foodstuffs as it does not carry the risk of chemical residue in the feed [33], and does not promote resistance to those chemicals in the bacterial population present in the wider world [43]. Methods like UAS technology that can clean with cold water, without heating, save significantly on the energy used for heating [8].

In this study, live imaging using EDIC/EF microscopy was chosen to evaluate the cleaning process rather than culture recovery since the latter does not show any remaining cells tightly adhered to the complex hay surfaces and also would not detect viable but nonculturable cells. Results showed that a 60-second exposure to an UAS resulted in an 87.94 ± 2.22% removal of the contaminants from hay, vastly exceeding the percentage removal seen with a cold water wash alone, which achieved a 21.85 ± 13.63% removal. These results show a significant difference between the efficacies of cleaning hay with UAS compared to a cold water wash, though there were variations observed in the results when surface topography is taken in to account, primarily following the water wash. This is likely a result of the surface topography being more complex with the grass-like hay (Fig. 6), creating more occluded spaces, which water wash is unable to clean as effectively and as easily as UAS. This is because ultrasonic cleaning is capable of cleaning contaminants trapped in cracks and crevices [13,33]. The result of which would be that hay with more occluded surface would exhibit a higher bacterial

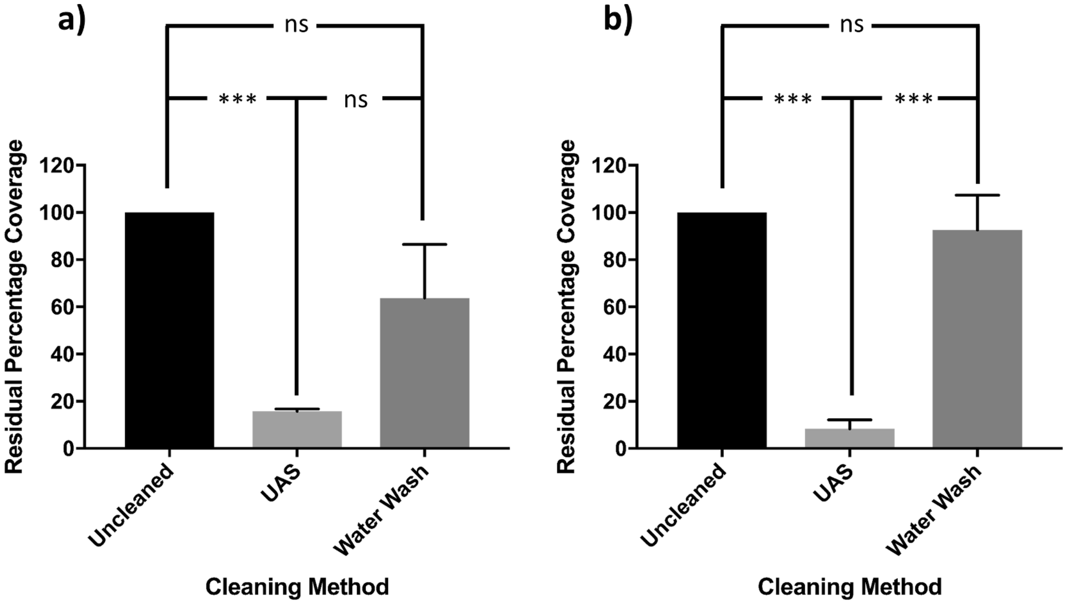

Fig. 5. Analysis of the EDIC microscopy images (n = 5 samples, each with 10 random fields of view) showing the percentage remaining on the sample after being cleaned using UAS and water. Samples have been categorised based on their topographical features into **(a)** straw-like samples and **(b)** grass-like samples. The uncleaned results are set to 100% by default and the error bars represent the SEM in each case. Significance results represent Tukey’s Multiple Comparisons Test, where non significance (ns) = $P > 0.05$, * = $P \leq 0.05$, ** = ≤ 0.01, *** = $P \leq 0.001$, and **** = $P \leq 0.0001$.

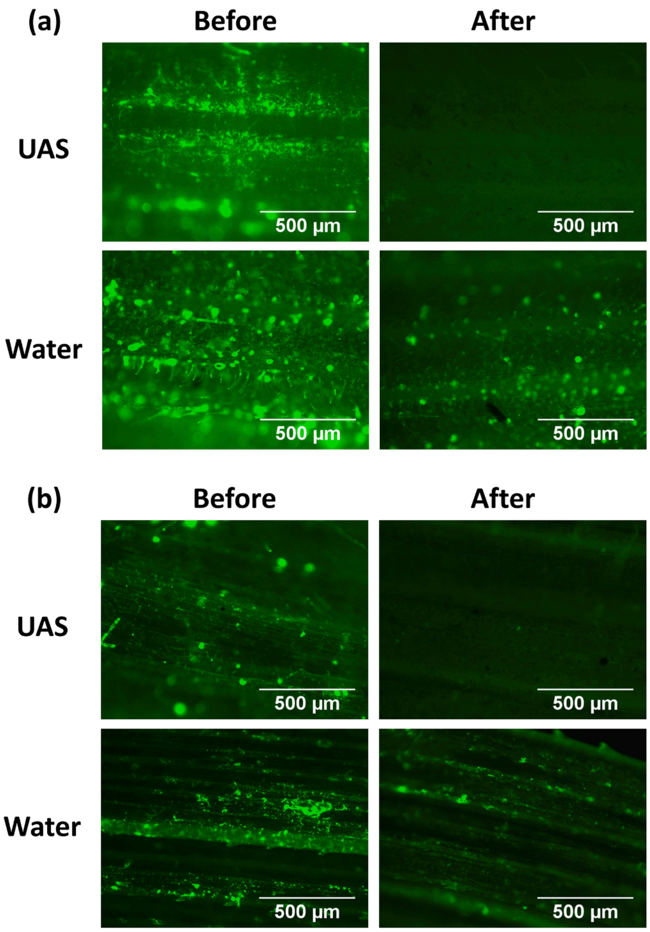

Fig. 6. (colour online) EDIC microscopy images of **(a)** Straw-like samples and **(b)** Grass-like samples at x100 magnification. Microbes are visualised as green fluorescent particles in the images as a result of staining with SYTO-9. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

load post-cleaning with the water-wash than its un-occluded counterpart, while UAS provided a more consistently effective clean regardless of the variation in surface topography of the of hay types. The increase in microbial load after a cold water wash as shown in Table 1 could be caused by the contaminants being redeposited onto the hay surface through the recirculating water system of the StarStream Mk 1 device setup. These phenomena were not observed in the samples cleaned with UAS. The SYTO-9 nucleic acid stain used in this study will not distinguish between live and dead cells on its own and therefore, we cannot comment on the fate of the bacterial cells washed with UAS, other than to quantify the percentage that each technique removed from the leaves.

5. Conclusion

Results suggest that UAS achieved effective removal of bacterial contaminant from hay and the cleaning mechanism of UAS can be adapted for the development of a new hay cleaning strategy that will take a lot less time to get an as effective clean. A key next step in using UAS to clean hay would be to use the current stream technology to develop a bulk-product cleaning strategy that which can reduce handling time and also remove bacterial and mould contaminant from the surface without damaging the hay.

CRediT authorship contribution statement

Weng Yee Chong: Investigation, Formal analysis, Writing - original draft. **Christian Cox:** Investigation, Formal analysis, Writing - original draft. **Thomas J. Secker:** Supervision, Methodology, Writing - original draft. **Charles W. Keevil:** Supervision. **Timothy G. Leighton:** Conceptualization, Supervision, Writing - original draft.

Declaration of Competing Interest

The inventor (T.G.L.) holds patents for UAS and StarStream in several countries, and is a Director and Inventor-in-Chief of Sloan Water Technology Limited, which owns the rights to the technology, but he has not taken pay or other remuneration from the company to date.

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