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Inactivation of *Listeria monocytogenes* ATCC7 644 Biofilms using Sodium Dodecyl Sulphate, Levulinic Acid and Sodium Hypochlorite Solution

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A study was done to assess the effectiveness of 200 ppm sodium hypochlorite (chlorine), 1% sodium dodecyl sulphate (SDS) and 0.5% levulinic acid in reducing *L. monocytogenes* ATCC7644 biofilms. 0.05% SDS and 0.5% levulinic acid were also used combined (mixture). After treatment with sanitizers, the biofilms were stored at 4°C for up to 72 hours and samples were tested at 0, 24, 48 and 72 hours. The contact times were varied to 1, 3, 5 minutes. Results revealed that biofilms were still viable after treatment with these sanitizers. There was no significance difference between storage times. Varying contact times from 1 to 3 minutes did not show a significance difference however there was a significance difference when the contact time was increased to 5 minutes. Non-adapted biofilms had highest log reductions compared to chlorine adapted and heat adapted biofilms. Treatment with chlorine was least effective in reducing viability of biofilms, followed by levulinic acid then a mixture of levulinic acid and SDS. SDS used alone had highest log reductions. Application of sanitizers at different contact times combined or individually may be successful in reducing biofilms in food manufacturing units. A careful selection of sanitizer for each specific pathogen may be required if sanitizers are to work effectively against biofilms.

Key words: Food safety, Fresh produce, *Listeria monocytogenes*, Biofilms, Adapted biofilms, Sanitizers, Food borne illnesses.

Biofilms are a community of microorganisms attached to a surface (Abee *et al.*, 2011). They are formed when a group of single celled organisms come together and then encased in an exo-polysaccharide matrix (Niemira, 2010). Their formation starts with motility to the surface, attachment, formation of clusters, development of differentiated structures, and dispersal (Wood *et al.*, 2011). Biofilms attach via appendages, such as fimbriae and flagella, and micro-colonies are formed by the production of microbial products including polysaccharides, proteins, lipids, and DNA and these play a structure-stabilizing and protective role in biofilm (Renier *et al.*, 2011). Environmental factors such as pH, water activity, temperature and nutrient composition of the food soil is important for the phenotypic transition of planktonic cells to sessile form (Belessi *et al.*, 2011). The resultant body exhibit different characteristics to the singular bacteria from which they were made (Bridier *et al.*, 2011) and can either be mixed species or one type of bacteria.

Biofilms are a concern in food manufacturing as their presence may lead to post

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processing contamination (Kim & Wei, 2007) since they attach to food preparation surfaces as well as equipment. It has been established through research that biofilms are resistant to chemical decontamination and sanitizers (Bridier *et al.*, 2011). The exopolysaccharide matrix formed by biofilms and cross linking of flagella and fimbriae creates a body that is difficult to penetrate and hence sanitizers cannot reach the internal layers (Bridier *et al.*, 2011). Mixed biofilms have also been implicated as being more resistant to sanitizers than their single specie biofilm (Abee *et al.*, 2011). Previous studies by Van der Veen & Abee (2011) found that a mixed biofilm of *Lactobacillus planturum* and *Listeria monocytogenes* was resistant to benzalkonium chloride and peracetic acid.

Resistance to sanitizers is also enhanced by stress-associated genes formed during biofilm formation (Abee *et al.*, 2011; Wood *et al.*, 2011). Studies have also shown that biofilms are capable of dispersing and their dispersal may be triggered by environmental or nutritional changes in their community (Wood *et al.*, 2011). Biofilms may also develop an adaptation characteristic due to repeated exposure to the biocides (Bridier *et al.*, 2011). These and other factors mentioned above have caused the control of biofilms to remain a challenge in food processing plants.

Among other pathogenic bacteria *L. monocytogenes* have also been known to form biofilms that are resistant to biocides (Bae *et al.*, 2012). *L. monocytogenes* can form single species biofilms, however in food manufacturing environments they are likely to form a mixed biofilm with other species either Gram positive or Gram negative bacteria (Van der Veen & Abee, 2011). *L. monocytogenes* have been known to pose serious threat to human health (Pan *et al.*, 2006). The elimination of *L. monocytogenes* biofilms in processing plants is critical for improving food safety. The objective of this study was to identify a sanitizer that can best reduce or eliminate *L. monocytogenes* ATCC7644 biofilms on tomatoes. The findings of this study will inform the food manufacturing bodies on the other potential sanitizers that could be tried to combat the long standing problem of biofilms thereby improving food safety.

MATERIALS AND METHODS

Bacterial strains

Listeria monocytogenes ATCC 7644 (Merck, South Africa) was used for this study. The strain was cultured in Fraser broth for 24 hours at 37º C and stored at 4º C (Ijabadeniyi *et al.*, 2011). Prior to each experiment, a fresh culture was prepared from the stock culture by sub culturing in Fraser broth for 24 hours at 37 º C, an 8 log cfu/ml culture of *L. monocytogenes* using McFarland Standards (Ji *et al.*, 2010).

Preparation of biofilms

Preparation of biofilms was carried out according to the method of Niemira (2010). Pre cleaned glass microscope slides were wrapped in foil paper and sterilised by autoclaving for 15 minutes at 121°C. Slides were aseptically placed into 50 ml centrifuge tubes containing 25 ml of tryptose soy broth (TSB-Oxoid Ltd, Wade Road, Basingstoke, Hants UK) using forceps. The solutions were inoculated with 200 µl of approximately 10^8CFU/ ml bacterial solutions and incubated at 37° C for 48 hours under static conditions. The same procedure was followed for heat adapted biofilms and chlorine adapted biofilms using heat adapted and chlorine adapted *L. monocytogenes* ATCC 7644.

Chemicals and chemical treatments

Sodium dodecyl sulphate (SDS), levulinic acid, sodium hypochlorite solution, all purchased from Merck, South Africa were tested, individually or combined. Contact times 1, 3 and 5 minutes were investigated. The chemicals were used as follows;
1% SDS individually
0.5% Levulinic acid individually
200 ppm Sodium hypochlorite solution individually and 0.5% levulinic acid/0.05% SDS combined and termed mixture.

Biofilm treatment with different sanitizer solutions

Following biofilm formation the slides were carefully removed from TSB solution using sterile forceps gripping the clean, dry upper portion of the slide. They were rinsed for 10 seconds under a stream of sterile distilled water to remove unattached cells. The slides were transferred into treatment solutions in other centrifuge tubes containing 25ml of test solutions (chlorine, levulinic acid, SDS and SDS/Lev mixture). Exposure times

were varied to 1, 3, 5 minutes. After treatment, the slides were then vigorously shaken in 25 ml of PB in a fresh, sterile 50-ml centrifuge tube. The suspension was serially diluted (1:10) in 0.1% buffered peptone water and enumerated for *L. monocytogenes* ATCC 7644. Samples of suspensions were also kept for 24, 48 and 72 hours for assessment.

Enumeration of *L. monocytogenes* biofilms

A method by Taormina & Beuchat (2001) was followed. Populations of *L. monocytogenes* ATCC 7644 biofilms were determined by surface plating serially diluted samples; 0.1 ml in duplicates on Listeria Selective Agar (Oxford formulation; Oxoid Ltd, Wade Road, Basingstoke, Hants UK). Plates were incubated for 24 hours at 37 º C after which colonies were counted.

Data analysis

Three trials were conducted for each experiment. Analysis of the data was performed using SPSS version 21 (IBM Statistics). Analysis of variance was conducted with repeated measures and Greenhouse Geisser correction to study the effect of contact time on the survival of *L. monocytogenes* ATCC 7644 biofilms and the effect of each sanitizer on the survival of *L. monocytogenes* ATCC 7644 at varied time intervals (0, 24, 48, 72 hours). The number of surviving colonies was plotted against contact time (1, 3, 5 minutes) and also against time interval (0, 24, 48, 72 hours). Log reductions for each contact time and sanitizer were also calculated and presented in a table. Pair wise comparison with Bonferroni adjustment was used to determine any significance difference between subjects.

RESULTS

Effect of sanitizer and contact times on the survival of non-adapted, chlorine adapted and heat adapted biofilms

The sanitizers tested were able to reduce the surviving colonies for non- adapted, heat adapted and chlorine adapted biofilms as seen by overall log reductions achieved as well as plotted marginal means. Increasing contact times reduced significantly the surviving colonies for non-adapted biofilms. Though varying contact times for 1, 3 and 5 minutes resulted in a significant fall in surviving colonies for non- adapted biofilms, ANOVA results with Greenhouse- Geisser correction showed that varying contact times from 1 minute to 3 minute did not cause significant reduction of colonies, while a significant fall in surviving colonies was achieved at 5 minutes. The case was different for heat adapted and chlorine adapted biofilms.

Overall log reductions

When exposed for 1 minute to 200 ppm chlorine, non-adapted biofilms were reduced by 1.73 log CFU/ml. A log reduction of 1.77 log CFU/ml and 1.80 log CFU/ml was noted after increasing contact time to 3 minutes and 5 minutes respectively. A mixture of 0.5% levulinic acid and 0.05% SDS (mixture) reduced the surviving colonies to 1.78 log CFU/ml, 1.80 log CFU/ml and log 2.86 CFU/ml after exposure for 1 minute 3 minute and 5 minutes respectively. Using 0.5% levulinic acid resulted in log reductions of 1.74 log CFU/ml, 1.75 log CFU/ ml and 2.0 CFU/ml after exposure for 1 minute, 3 minutes and 5 minutes. SDS reduced surviving colonies by 1.79 log CFU/ml 1.86 log CFU/ml, 3.54 log CFU/ml for 1, 3, 5 minutes respectively (Table 1[1]). The chlorine adapted and heat adapted biofilms had lower log reductions compared to non- adapted biofilms. This showed

Table 1. Log reductions (CFU/ ml) for all sanitizers at 1, 3, 5 minutes; [1] Non- adapted biofilms [2] chlorine adapted biofilms [3] heat adapted biofilms

Overall log reduction non adapted biofilms [1]			
Sanitizer	1 minute	3 minutes	5 minutes
Chlorine	1.73	1.77	1.80
Mixture	1.78	1.80	2.86
Levulinic	1.74	1.75	2.00
SDS	1.79	1.86	3.54
Overall log reduction chlorine adapted biofilms [2]			
Sanitizer	1 minute	3 minutes	5 minutes
Chlorine	1.69	1.70	1.77
Mixture	1.74	1.77	2.09
Levulinic	1.73	175	1.80
SDS	1.78	1.88	3.17
Overall log reduction heat adapted biofilms [3]			
Sanitizer	1 minute	3 minutes	5 minutes
Chlorine	1.69	1.70	1.70
Mixture	1.74	1.77	2.33
Levulinic	1.73	1.75	1.80
SDS	1.78	1.80	3.32

that the adapted biofilms had developed a resistance to the treatments and hence they survived more due to adaptive response.

Chlorine adapted biofilms were reduced by 1.69 log CFU /ml, 1.70 log CFU/ml 1.77 log CFU/ ml when exposed to 200 ppm chlorine for 1, 3, 5 minutes respectively. Log reductions for levulinic acid were relatively lower than chlorine with 1.73 log CFU/ ml, 175 logCFU/ ml and 1.80 log CFU/ ml respectively. A mixture of 0.5% levulinic and 0.05% SDS performed better than levulinic used individually with log reductions of 1.74 log CFU/ ml, 1.77 log CFU/ml and 2.09 log CFU/ml for 1, 3, 5 minutes respectively. SDS had highest log reductions of 1.78 log CFU/ml, 1.88 log CFU/ml and 3.17 log CFU/ml for the selected contact times (Table 1[2]).

The log reductions for heat adapted biofilms were more or less similar to those of chlorine adapted biofilms. Log reductions of 1.69 log CFU/ ml, 1.70 log CFU/ml and 1.70 log CFU/ml were achieved when heat adapted biofilms were subjected to 200ppm chlorine for 1, 3, and 5 minutes respectively. A mixture of 0.05% SDS and 0.5% levulinic led to log reductions of 1.74 log CFU/ml, 1.77 log CFU/ml and 2.33 log CFU/ml while exposure to 0.5% levulinic acid individually achieved 1.73 log CFU/ml, 1.75 log CFU/ml and 1.80 CFU/ml for 1, 3, 5 minutes respectively. High log reductions were achieved by use of 1% SDS (Table 1[3]).

The marginal means of surviving colonies were plotted against sanitizers for each contact times. The results presented in figure 1 show that among the sanitizers tried chlorine was least

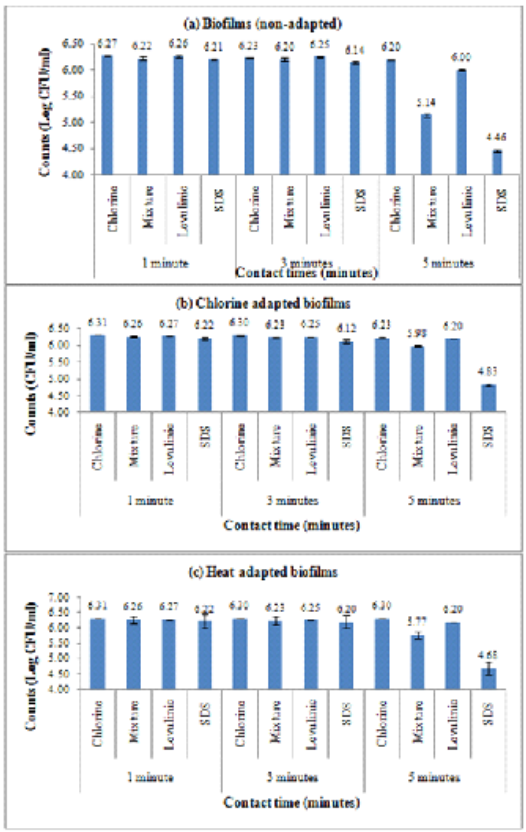

Fig. 1. Marginal means of surviving colonies for non-adapted (a), chlorine adapted (b) and heat adapted biofilms (c). The low mean counts associated with chlorine show that it was least effective among other sanitizers.

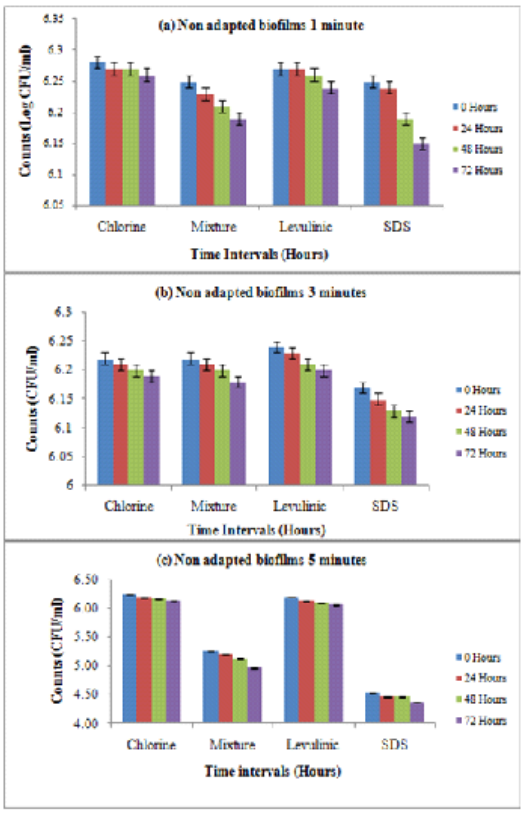

Fig. 2. Survival of *L. monocytogenes* ATCC7644 biofilms (non- adapted) followed by sanitiser treatment for 1minute (a), 3 minutes (b) and 5 minutes (c)

effective as it had highest means of surviving colonies regardless of whether the biofilms were adapted or not.

Effect of sanitizer treatments and storage time on the survival of non-adapted, chlorine adapted and heat adapted biofilms

The action of chlorine, SDS, levulinic and mixture was significantly different for all tested bacteria. As reported earlier in this article, chlorine was least effective. Increasing storage times (time intervals) from 0 hours to 72 hours did not cause a significant fall in surviving colonies, according to the findings of this research. Though there was a reduction in surviving colonies for up to a storage time of 72 hours, the reductions were not significantly different. Figure 2, 3 and 4 shows the trends.

DISCUSSION

Sanitizers are chemical agents used to inactivate bacteria in food processing units. Previous studies have shown that biofilms are resistant to sanitizers (Abee *et al.*, 2011; Renier *et al.*, 2011; Wood *et al.*, 2011). In this study, there were surviving bacteria after treatment with sanitizers. Chlorine had the lowest log reductions followed by levulinic acid then mixture; while SDS had the highest log reductions.

The ineffectiveness of chlorine in eradicating pathogens has been previously reported (Allende *et al.*, 2009; Gil *et al.*, 2009; Kim *et al.*, 2009; Mahmoud *et al.*, 2007). Previous studies have also shown that SDS or levulinic when used alone does not achieve significant results

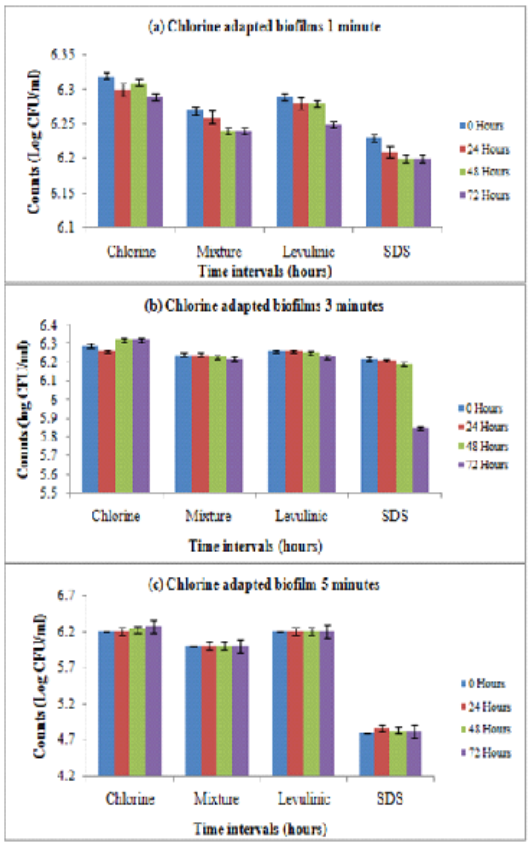

Fig. 3. Survival of *L. monocytogenes* ATCC7644 biofilms (chlorine adapted) followed by sanitiser treatment for 1minute (a), 3 minutes (b) and 5 minutes (c)

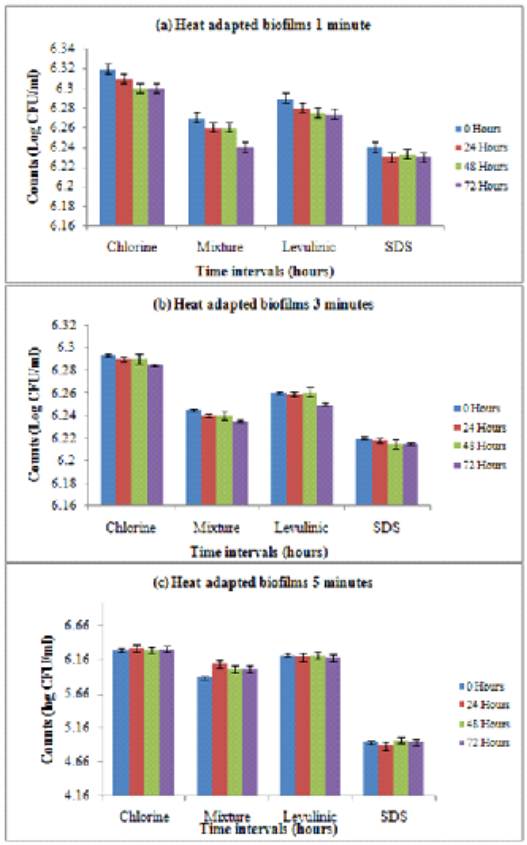

Fig. 4. Survival of *L. monocytogenes* ATCC7644 biofilms (heat adapted) followed by sanitiser treatment for 1minute (a), 3 minutes (b) and 5 minutes (c)

(Cannon *et al.*, 2012; Zhao *et al.*, 2009). In this study a concentration of 0.05% SDS mixed with 0.5% levulinic acid could not achieve total reduction of biofilms for the entire storage period. Other studies using higher concentrations 3% levulinic acid and 2% SDS achieved a reduction of *Salmonella* from 19% before treatment to 1% after treatment, coliform counts were reduced from 6 - 8 to 2 - 4 log CFU/9 cm^2, and aerobic plate counts were reduced from 7 - 9 to 4 - 6 log CFU/9 cm^2 (Zhao *et al.*, 2011). A 5% levulinic and 2 % SDS was also tried effectively on influenza virus (Aydin *et al.*, 2013). These findings suggest that higher concentrations of sanitizers may be required to reduce biofilms effectively. However, higher concentrations may interfere with sensory properties of food items and it could be hazardous.

From the results, it can be concluded that the non-adapted biofilms were more responsive to sanitizer treatment compared to chlorine adapted and heat adapted biofilms. The bacterial counts for chlorine adapted biofilms were more or less similar to those of heat adapted biofilms, with heat adapted counts slightly higher than chlorine adapted. This suggests that chlorine adapted biofilms and heat adapted biofilms were more resistant to sanitizers. From these findings it can be concluded that chlorine, levulinic acid and SDS are not able to eradicate adapted biofilms. Other researchers have also reported on the biofilm resistance to sanitizer. Machado *et al.* (2012) using benzalkonium chloride found that adapted biofilms of *Pseudomonas aeruginosa* and *Escherichia coli* maintained their mass and activity after treatment while Stopforth *et al.* (2002) found no differences between previously acid-adapted and non-adapted *L. monocytogenes* with regard to sensitivity to sanitizers using sodium hypochlorite and quaternary ammonium compound.

Apart from the sanitizers mentioned above biofilms have been reported to be resistant to benzalkonium chloride, peracetic acid and nisin (Ibusquiza *et al.*, 2011). Cruz & Fletcher (2012) found that out of the twenty one sanitizers tested against *L. monocytogenes* in biofilm, only the peroxyacetic acid, chlorine dioxide and acidified sodium chorite-based products gave a 5 log CFU/ml decrease. It is well known through research that biofilms are more resistant than their planktonic counterparts (Abee *et al.*, 2011; Bridier *et al.*, 2011; Van der Veen & Abee, 2011; Gandhi & Chikindas, 2007; Kim & Wei, 2007; Joshua *et al.*, 2006; Pan *et al.*, 2006) and that mixed biofilms are more resistant that single species biofilms (Van der Veen & Abee, 2011). A use of combined methods could help in reducing viability of biofilms. Other researchers suggested mechanical abrasion with subsequent spray applications of sanitizer to reduce biofilm and non-biofilm populations of *L. monocytogenes* from stainless steel surface (Chambliss-Bush, 2012).

Resistance of biofilms to sanitizers is attributed to many factors. It can be intrinsic, genetically acquired or phenotypically induced. According to Lambert & Johnston (2001) sanitizer effectiveness can be impaired by the presence of organic molecules such as proteins, nucleic acid and carbohydrates. This is because sanitizers are highly chemically reactive and may thus interact with the organic molecules. Ganeshnarayan *et al.* (2009) mentioned the presence of electrostatic forces as having a negative effect on movement of cationic surfactants across negatively charged biofilms thereby reducing biocide effectiveness. Hydrophobic interactions due to long Carbon-chains have also been implicated in reducing sanitizer effectiveness (Habimana *et al.*, 2010; Sandt *et al.*, 2007). Leriche *et al.* (2003) reported that *Staphylococcus sciuri* was protected from chlorine treatment due to a mixed biofilm it formed with a more resistant strain of *Kocuria spp.*

Increasing contact time decreased significantly the surviving colonies of *L. monocytogenes.* This is evidence that, the longer the bacteria are exposed to chemicals the greater the chances of reducing their survival. Several researchers agree that increasing contact time increases effectiveness of sanitizers(Møretrø *et al.*, 2012; Ding *et al.*, 2011; Mattson *et al.*, 2011; Park *et al.*, 2011) as more time is allowed to penetrate the three dimensional aggregate of biofilms.When biofilms where stored up to 72 hours, their numbers did not decrease significantly. These results suggest that biofilms were either able to recover during the storage period or continued to multiply since sanitizers had not completely inactivated them.

CONCLUSION

Sanitizers do not completely destroy *L. monocytogenes* biofilms. Adapted biofilms are more resistant to sanitizers compared to non-adapted biofilms. A contact time of 5 minutes is not enough to eradicate biofilms and hence a higher contact time or increase in concentration of sanitizers may show better results.

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