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Enduring pathogenicity of African strains of *Salmonella* on plastics and glass in simulated peri-urban environmental waste piles

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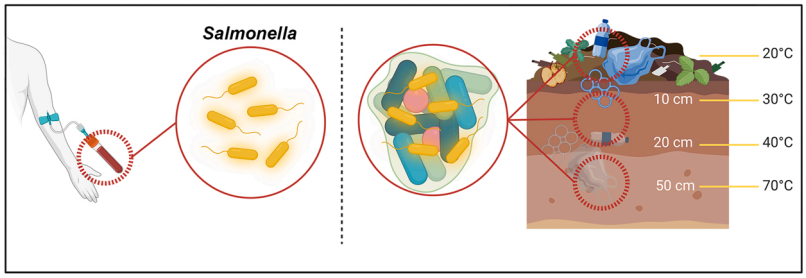
HIGHLIGHTS

- African strains of pathogenic *Salmonella* can persist on environmental plastic waste.
- Temperature affects the duration of persistence of *Salmonella* in the plastisphere.
- *Salmonella* retains virulence and pathogencitiy following recovery from the plastisphere.
- Pathogens colonising plastic pose a heightened environmental and public health risk.

GRAPHICAL ABSTRACT



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ABSTRACT

In low- and middle-income countries, plastic has become a major constituent of landfills and urban dump sites. Environmental plastic pollution can also provide a novel surface for the formation of microbial biofilm, which often includes pathogenic bacteria and viruses. Here, under conditions simulating a peri-urban waste pile typical of an African informal settlement, we aimed to determine if pathogenic *Salmonella* spp. can retain their virulence following a prolonged period of desiccation on the surfaces of environmental plastic and glass. We show that clinically (and environmentally) relevant strains of *Salmonella* including *S.* Enteritidis, *S.* Typhimurium and *S.* Typhi can persist on plastic and glass for at least 28-days and that temperature (which increases with the depth of an urban waste pile) is a key determinant of this survival. All three strains of *Salmonella* retained their pathogenicity (determined by using a *Galleria mellonella* model of infection) following their recovery from the plastisphere indicating that plastics in the environment can act as reservoirs for human pathogens and could facilitate their persistence for extended periods of time. Pathogens colonising environmental plastic waste therefore pose a heightened public health risk, particularly in areas where people are frequently exposed to plastic pollution.

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1. Introduction

Access to improved sanitation remains poor in many African cities, due to rapid urbanisation combined with inadequate waste management and sanitation infrastructure [1,2]. Despite wide-scale recognition of the need for more significant government investment in solid waste management, this remains a low priority area for funding [3,4]. Consequently, in the absence of regular, effective collection services, communities are forced to either burn their waste (significantly reducing air quality) or dump it directly into the environment leading to uncontrolled urban dump sites [5,6]. In addition to attracting vermin, these dump sites can act as reservoirs for human faecal pathogens [7] and thus pose a significant public health risk for those directly interacting with this waste, such as, informal waste-pickers [8].

Plastic pollution is increasing concurrently with both economic development and urbanisation in many cities in sub-Saharan Africa [9], and has now become a major constituent of landfills and urban dump sites [10]. During the rainy seasons, plastic waste often blocks urban drainage systems, which can lead to localised flooding and an increased risk of human exposure to raw sewage and thus the spread of waterborne pathogens within highly populated areas [11,12]. Distinct microbial populations can colonise environmental plastic debris in what is collectively known as the 'plastisphere' [13], with increasing evidence that environmental plastic waste can become colonised with human pathogenic bacteria and viruses [14-16].

In Africa, *Salmonella* is the most frequently isolated pathogen in hospitalized patients diagnosed with community-onset blood stream infection (BSI) [17,18]. Case fatality rates of approximately 25% in children to 50% in adults results in almost 50,000 deaths each year [19]. The aetiologic agents of invasive *Salmonella* include both the classically invasive *Salmonella* serovar, *S. enterica* serovar Typhi (*S.* Typhi) and several non-typhoidal *Salmonella* (NTS) serovars such as *S. enterica* serovar Typhimurium (*S.* Typhimurium); *S. enterica* serovar Enteritidis (*S.* Enteritidis); and distinct lineages geographically restricted to sub-Saharan Africa [20,21].

Most *Salmonella* serovars are pathogenic to humans; however, the zoonotic potential of NTS means that animals can act as asymptomatic carriers [22,23]. This is important because domestic, feral, and wild animals are attracted to dumpsites, particularly in low- and middle-income countries (LMICs) [7,24], and will often consume human faecal material, e.g., from open defecation or contained in diaper materials [25,26]. Animal faeces have been identified as a significant reservoir of enteric pathogens, including *Salmonella* spp. [22], and the indirect transmission of *Salmonella* from animals to humans is possible due to the ability of many *Salmonella* serovars to survive in the environment. *Salmonella* spp. have developed multiple strategies to enhance their survival under environmental conditions [27], and can persist for extended periods on different materials, including plastics, following desiccation, (e.g., [28]). With increasing evidence that human pathogenic bacteria can retain their virulence on the surfaces of plastics [16], there is an urgent need to understand the potential for clinically important strains of *Salmonella* to remain pathogenic on environmental plastic waste and thus present an additional public health risk in urban and peri-urban community settings.

The aims of this study therefore, were to: (1) quantify the potential for clinically important groups of *Salmonella*, including *S.* Typhimurium , *S.* Enteritidis and *S.* Typhi to colonise and persist on plastic waste under environmental conditions; and (2) determine the pathogenicity of these strains following their recovery from the plastisphere. To address this, we have quantified *Salmonella* survival on two types of plastic (polyethylene [HDPE] and polypropylene [PP]), and glass, under conditions characteristic of an urban waste pile in sub-Saharan Africa. To determine the state of pathogenicity of these recovered *Salmonella* cells, a *Galleria mellonella* model of infection was used to assess any changes in pathogenicity.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The pathogenic strains of *Salmonella* used in this study were, *S.* Enteritidis strains D7795 (East African lineage) and CP255; and African *S.* Typhimurium strain of MLST ST313 D23580; and attenuated *S.* Typhi strain ZH9 (Table 1). Prior to use, isolates were grown in Luria-Bertani (LB) broth (Invitrogen, UK) at 37 °C and shaking at 120 rpm, unless otherwise stated. Following recovery from plastic or glass surfaces, isolates were grown on Bismuth Sulphite agar (Merck, Germany), prepared according to the manufacturer's instructions.

2.2. Biofilm assay

Biofilms were generated and quantified as described previously [32]. Briefly, overnight cultures of each *Salmonella* strain were grown in LB at 37 °C. The following morning, cultures were diluted 1:100 into either fresh LB or fresh surface water (final volume 100 μl) in a 96-well plate, and covered with a plate seal, and placed inside a Tupperware box containing damp tissue paper to maintain humidity. Surface (river) water was collected from the Allan Water (Dunblane, Scotland, UK). Boxes were placed at 20, 30 or 40 °C for 48 h. Biofilms were enumerated using the crystal violet method [32]. All experiments were conducted in biological triplicate.

2.3. Formation of natural biofilm on glass, high density polyethylene and polypropylene

One hundred and eight metal frames containing either blue high-density polythene (HDPE) (20 μm; Thali outlet, Leeds, UK; 36 frames), blue foil-backed polypropylene (PP) crisp packets ('Wotsits', Walkers Crisps, UK; 36 frames), or untreated glass microscope slides (Academy, UK; 36 frames) were prepared in the same way as previously described [16]. Briefly, materials were housed in bespoke high-grade stainless--steel frames (200 mm × 140 mm x 1.5 mm) and submerged in surface water (subsequently called 'SW') to allow generation of a natural biofilm. Eighteen frames (six each of glass, HDPE and PP) were submerged in one of six replicate glass tanks containing the SW and aerated continuously using a Pond Air Pump (Swell, UK). The tanks were covered to prevent evaporation and all frames submerged for 96 h at ambient room temperature (ca. 18–21 °C) to allow a natural biofilm to develop on each material. Conductivity, pH, and turbidity of the SW were measured using a portable probe (Combo pH and EC, Hanna Instruments Ltd., UK) and a HI-88703–02 Bench Top Turbidity Meter (Hanna Instruments Ltd., UK) (Supplementary Table S1).

Table 1
Salmonella strains used in this study.

Strain	Type	Isolated	Location	Source	Reference
D7795	*S.* Enteritidis	1998	Blantyre, Malawi	Paediatric blood	[20]
CP255	*S.* Enteritidis	1991	DRC (Zaire)	Paediatric blood	[29]
D23580	*S.* Typhimurium	2004	Blantyre, Malawi	Paediatric blood	[30]
ZH9	*S.* Typhi	GM* in 2002	United Kingdom	Derivative of Ty2 (isolated from patient with typhoid fever)	[31]

* GM: Genetically modified, Ty2 Δ*aroC* Δ*ssaV*

2.4. Inoculation of frames

Salmonella cultures were grown overnight in LB and then diluted 1:100 into fresh, pre-warmed LB (to 37 °C) and grown to an $OD_{600\ nm}$ of 0.2. During this growth period, frames were removed from the tanks and air dried for at least 3 h, or until visibly dry. Once cultures had reached an $OD_{600\ nm}$ of 0.2, 30 ml were pelleted by centrifugation (4000 rpm, 10 min, 4 °C). Human faecal material (which had been stored frozen, and subsequently sterilised by autoclaving after thawing) was added to 40 ml of SW at a concentration of 10 mg/ml and thoroughly homogenised by vortexing (this faecal suspension is subsequently referred to as 'FS'). Bacterial pellets were then resuspended in 12 ml FS. Frames were reassembled, and 300 μl of the bacterial FS pipetted onto each of the six squares in each replicate frame. The concentration of the inoculum was approximately 1×10^7 bacterial cells, with concentrations comparable between isolates and replicates (data not shown). Frames were then placed into a sealed plastic box (19 L; 375 ×255×290 mm) in which a humid environment (85% relative humidity) was generated through the addition of moistened paper towels (25 ml water per box). Boxes were placed into incubators at either 20, 30 or 40 °C as representative temperatures recorded within an active waste pile (Fig. 1); every seven days, an additional 100 ml of water was added to each box, in order to ensure a humid environment was maintained. Glycerol stocks (40%) of each initial inoculum were made and stored at − 80 °C. The exact concentration of bacteria in each inoculum was determined retrospectively by serial dilution and enumeration of colony forming units (CFU).

2.5. Persistence of Salmonella isolates

Seven, 14, 21 and 28-days after inoculation (DAI), replicate frames of each material were removed from the incubator, and 300 μl sterile PBS (phosphate buffered saline) added to each square and left at room temperature, for 15 min. Using a sterile plastic loop, the drop of PBS was agitated and the surface gently scraped to disperse the biofilm. From this suspension, 20 μl was serially diluted in PBS and plated onto selective agar (Bismuth sulphite; Merck, Germany) for *Salmonella* enumeration. The remaining suspension was added 1:1 with 80% glycerol and stored at − 80 °C for subsequent *Galleria mellonella* infection assays. Confirmatory PCR was conducted on isolates to be used for infection of *G. mellonella* larvae to validate the selective media used, and to ensure the isolates were *Salmonella* spp. Colony PCR of the *ttr* gene (encoding tetrathionate reductase; *ttr*_for: 5′-CTCACCAGGAGATTACAACATGG-3′; *ttr*_rev: 5′-AGCTCAGACCAAAAGTGACCATC-3′ [33]) was conducted retrospectively following enrichment of cultures from glycerol stocks in LB broth, followed by plating on solid BSA media (Fig. S1). The *ttr* gene is commonly used as a PCR gene target to detect and identify *Salmonella* due to its presence in all *Salmonella* spp. [33,34].

The extraction efficiency of the technique was assessed to ensure equal amounts of bacteria could be extracted from each material. Briefly, natural biofilms were formed on each material (HDPE, PP and glass) contained within frames, as described above. The frames and materials were allowed to air dry, before approx. 5×10^8 CFU of strain D23580 (used as a model organism) suspended in FS was added to the surfaces of the three materials. Frames were incubated at 30 °C for 48 h. Following this, 300 μl of PBS was added, and the sample left at room temperature for 15 min. The solution was agitated using a sterile plastic loop and 20 μl was used for serial dilution and enumeration.

2.6. Challenge of Galleria mellonella

G. mellonella larvae (Livefood, Axbridge, UK), were kept in the dark at 15ºC and used a maximum of one week after their delivery. Healthy larvae, measuring from 2 to 2.5 cm in length and showing no signs of melanisation were used for all experiments.

To assess the pathogenicity of each *Salmonella* strain at the concentration and metabolic state it was in at the time of recovery from each material, 10 μl of glycerol stock was injected into groups of 10 larvae, directly into the hemocoel via the last right pro-limb. For comparison, the initial inoculum that was added to the frames at day 0 and the control faecal material not containing the *Salmonella* was included. Following challenge, larvae were placed in an incubator at 37 °C and survival assessed for 72 h, with larvae considered dead when non-responsive to touch. Experiments were conducted in biological triplicate. For all challenge experiments, an inoculation of PBS was used as a negative control ($n = 10$ larvae per experiment) to account for mortality caused by physical injury or infection by a contaminant. Furthermore, inoculation of PBS containing 40% glycerol (PBS-G) was used to account for any toxicity related to the glycerol content of the stocks.

Additionally, using a pure culture of each *Salmonella* strain, a dilution series was generated for challenge into *Galleria* larvae, to determine how infectious dose related to survival of *Galleria* larvae. Concentrations of 10-fold increments ranged from 1×10^2 to 10^9 cells. *Salmonella* cultures were grown in LB, washed, and resuspended in PBS as described above; and *Galleria* were challenged and monitored as described above.

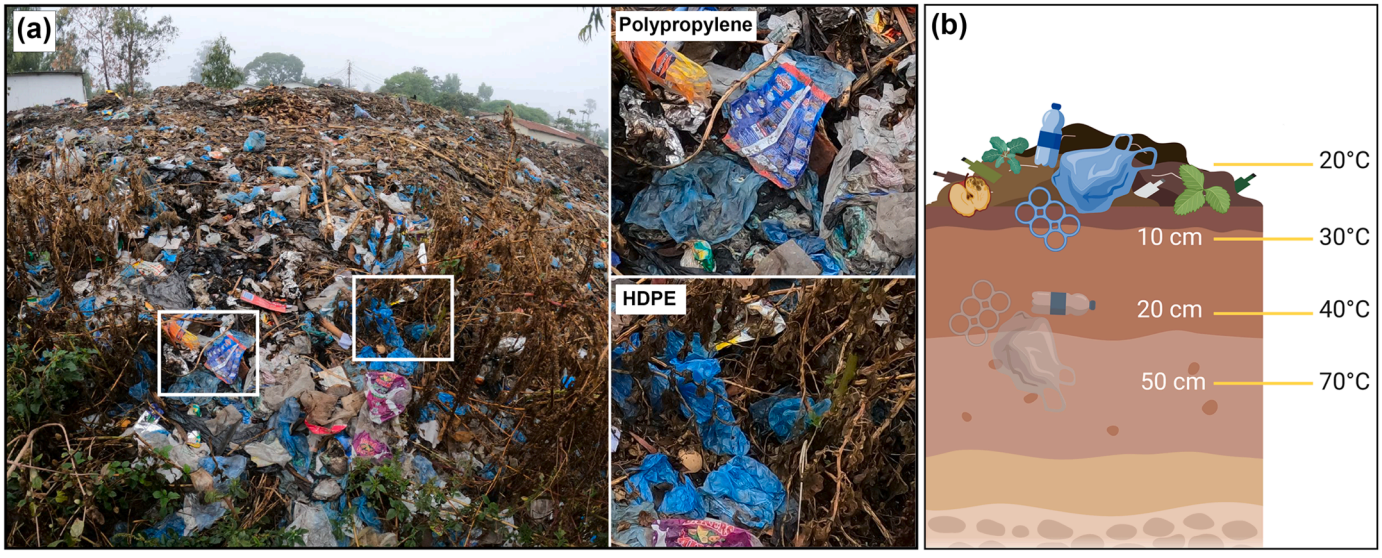

Fig. 1. Urban mixed waste piles typical of African informal settlements. (**a**) Waste pile in the informal settlement of Ndirande, Malawi (June 2022) with enlarged images showing examples of foil backed PP, and HDPE; (**b**) cross-sectional diagram showing the increase in temperature with depth.

Plots of percentage survival vs. challenge dose were generated allowing for a comparison with the concentrations of *Salmonella* recovered at each time point.

2.7. Statistical analysis

Statistical analyses were conducted using GraphPad Prism Software. Kruskal-Wallis test with Dunn's post-hoc tests were used to compare the survival of *G. mellonella*. *P* values < 0.05 were considered significant. To calculate die off rates, CFU counts were normalised by transforming to $\log_{10}$ CFU/ml. Linear regression analysis, carried out in Minitab version 18 (Minitab Inc.; State College, PA, USA), was used to establish relationships describing the pattern of bacterial decline, and subsequently used to determine the die-off characteristics of *Salmonella* under the different treatments using the same approach as Afolabi et al., (2020). The decrease in bacterial concentrations followed a linear decline over time, and so a log-linear regression model was fitted to the $\log_{10}$ transformed data and is described by the equation:

$$\mathrm{Log}_{10}(C) = \mathrm{Log}_{10}(C_0) - kt$$

where C_0 is the cell concentration at $t = 0$ and k is a die-off rate constant (d^{-1}). Using the log-linear model, the % decrease in bacterial concentration per unit time is constant. Decimal reduction times (*t90*-values; the number of days to reduce viable bacteria by 90%) were calculated based on the decline rates for populations following a log-linear die-off profile. Analysis of variance (ANOVA) was used to assess the effect of treatments on k values, and Tukey post-hoc tests used for mean comparisons. Furthermore, two- and three-way ANOVAs were conducted using SPSS (v. 28) to analyse the combined effects of variables (strain, temperature and material).

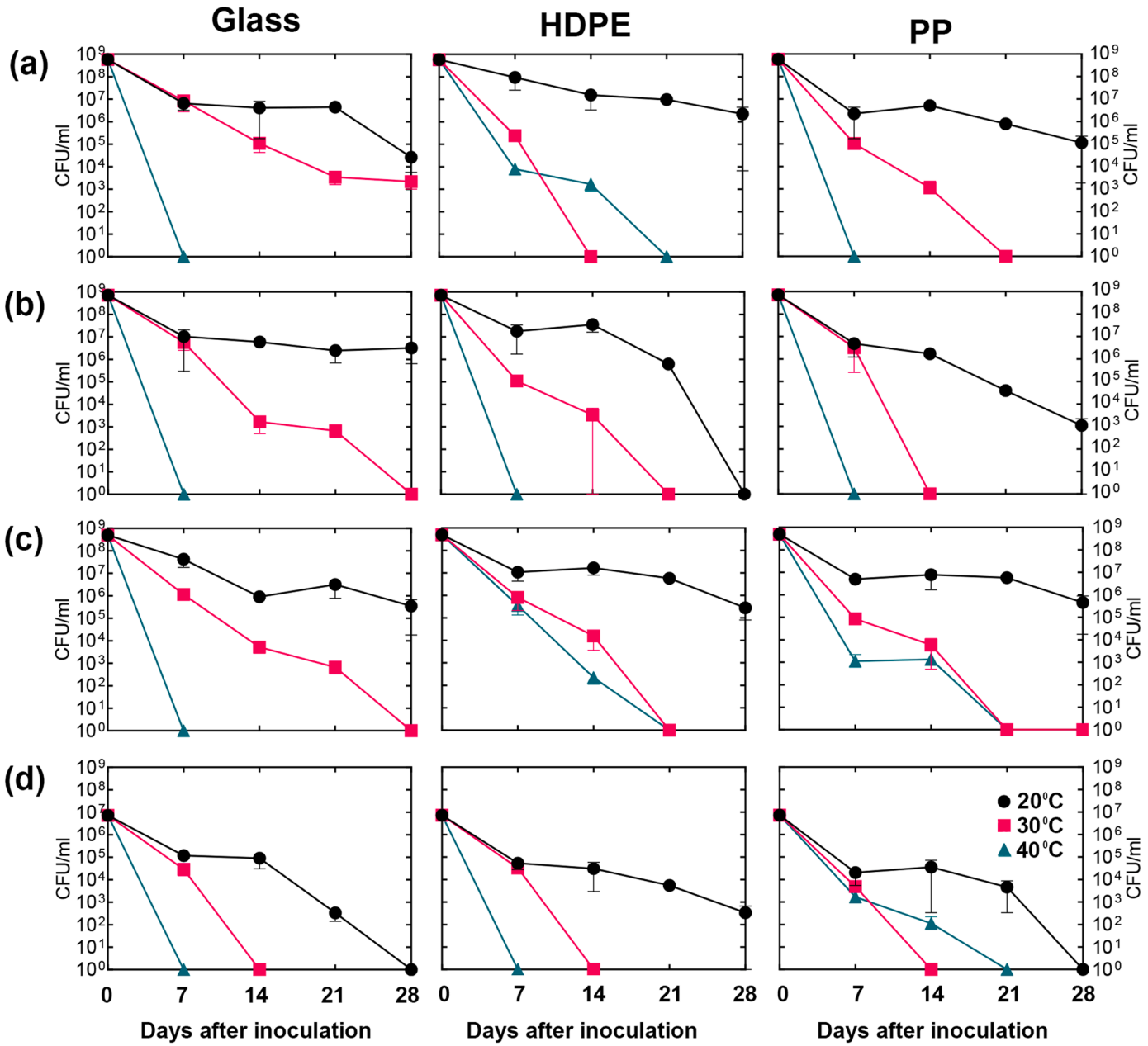

Fig. 2. The effect of temperature on the persistence of *Salmonella enterica* spp. Persistence on glass, HDPE, and PP of, (a) *S.* Enteritidis [D7795]; (b) *S.* Enteritidis [CP255]; (c) *S.* Typhimurium [D23580]; and (d) *S.* Typhi [ZH9] at 20 °C, 30 °C and 40 °C. Experiments were conducted in biological triplicate and data points represent the mean ± SE.

3. Results

3.1. The effect of temperature on Salmonella persistence on glass, HDPE, and PP

To ensure that our extraction method for recovering *Salmonella* isolates from the different materials did not bias our results, we examined the quantity of bacteria recovered from each surface after 24 h inoculation. There were no significant differences in total cell numbers of bacteria recovered from glass ($1.52 \times 10^6 \pm 6.8 \times 10^4$), HDPE ($1.61 \times 10^6 \pm 6.8 \times 10^4$) or PP ($2.0 \times 10^6 \pm 2.4 \times 10^5$), demonstrating that the technique did not favour extraction of bacterial cells from one material type over another.

At 20 °C, all strains survived for at least 21-days on glass, HDPE and PP, with *S.* Enteritidis strain D7795 and *S.* Typhimurium strain D23580 surviving for 28-days on all materials (Fig. 2). At 30 °C however, most strains were below the limit of detection beyond day 21 on any material, with only *S.* Enteritidis strain D7795 surviving for 28 days on glass, at a relatively low concentration (2.2×10^3 CFU/ml). At 40 °C, no strains were recovered from glass at the first sampling point (7 days); however, both *S.* Enteritidis strain D7795 and *S.* Typhimurium strain D23580 were recovered from HDPE at day 14; and *S.* Typhimurium strain D23580 and *S.* Typhi strain ZH9 were recovered from PP on day 14.

3.2. Survival rates of Salmonella are more dependent on temperature than substrate

Log linear regression models were applied to all replicates (R^2 ranged from 0.473 to 1.000) to determine modelled linear decline rate constants (*k)* and decimal reduction times (D-values) (Table 2; Fig. S2). Temperature significantly affected the persistence of each *Salmonella* strain, with die-off of most *Salmonella* strains more rapid at 40 °C than at 20 °C, on glass, HDPE and PP ($P < 0.001$), as indicated by the increasing linear decline values (*k*; day^{-1}). However, the rate of decline of *S.* Enteritidis isolate D7795 and *S.* Typhimurium isolate D23580, on HDPE, was not significantly affected by temperature ($P > 0.05$). The material type did not impact the survival rate of each *Salmonella* strain, with no significant differences between glass, HDPE and PP. However, when analysed in conjunction with temperature ($P < 0.001$) or strain ($P < 0.001$) significant differences were observed. Furthermore, there was a statistically significant three-way interaction between temperature, strain and material ($P < 0.001$). Additionally, temperature appeared to effect biofilm forming ability, with each strain forming more biofilm at lower temperatures. There were significant differences between strains, with the exception of strain CP255 which formed less biofilm than strains D23580, D7795 and ZH9 at 20 °C (Fig. S3).

3.3. Salmonella strains retain virulence and pathogenicity following recovery from plastisphere

Injection of FS and of PBS-G did not cause significant mortality to the *Galleria* larvae (Fig. S4), indicating that the mortality following infection with material recovered from the glass, HDPE and PP was due to the *Salmonella* and not the background material.

In all cases (irrespective of material type and temperature), each strain was pathogenic towards the *Galleria* larvae, with *S.* Enteritidis isolates D7795 and CP255, and *S.* Typhimurium isolate D23580 killing almost 60% of *Galleria* within 48 h, and 80% within 72 h. *S.* Typhi isolate ZH9, was less pathogenic, killing 40% of *Galleria* by 48 h, and 50% by 72 h (Fig. 3).

For isolates D7795, CP255 and D23580, the temperature at which the material had been incubated did not influence their pathogenicity. In most cases, no significant differences in pathogenicity were observed between isolates recovered from each material at 20 °C and 30 °C, relative to the initial inoculum. Isolates of *S.* Typhimurium recovered at 20 °C were significantly less pathogenic than those recovered at 30 °C, 24 h post inoculation (hpi; $P < 0.05$), although by 48 hpi there was no difference between these isolates. The pathogenicity of *S.* Typhi isolate ZH9 however, was affected by temperature: at 24, 48 and 72hpi, the pathogenicity of *S.* Typhi isolate ZH9 recovered from the glass at 30 °C was significantly less than that of the initial inoculum and ZH9 recovered at 20 °C ($P < 0.05$). Similarly, ZH9 recovered from HDPE at 20 °C, and PP at 20 °C and 30 °C were significantly less pathogenic towards the *Galleria* larvae than the initial inoculum ($P < 0.05$).

Comparison of expected survival rates of *Galleria* larvae versus actual survival rates following infection with material recovered from HDPE, PP and glass revealed that in most cases, virulence increased following recovery from the plastisphere. Also, the infectious dose was not always indicative of the virulence capabilities of each isolate, as in some cases there was a high death rate of *Galleria* even when challenged by samples containing relatively few *Salmonella* cells (Supplementary Table S2; Fig. S3).

Table 2
Linear decline rates and decimal reduction times for *Salmonella* spp.

Strain	Treatment		*k* (day^{-1})		D-value (days)	R^2
			Mean[a]	SEM		
***S.* Enteritidis (D7795)**	**Glass**	20 °C	0.106	0.011	7.5	0.715
		30 °C	0.207	0.057	4.3	0.906
		40 °C	0.757	0.218	1.3	0.977
	HDPE	20 °C	0.122	0.041	8.1	0.811
		30 °C	0.489	0.002	1.6	0.984
		40 °C	0.364	0.064	2.3	0.940
	PP	20 °C	0.109	0.013	7.4	0.736
		30 °C	0.429	0.061	1.9	0.986
		40 °C	0.978	0.004	0.8	1.000
***S.* Enteritidis (CP255)**	**Glass**	20 °C	0.067	0.020	13.4	0.473
		30 °C	0.271	0.037	3.0	0.958
		40 °C	0.987	0.005	0.8	1.000
	HDPE	20 °C	0.208	0.003	3.8	0.764
		30 °C	0.432	0.061	1.9	0.986
		40 °C	0.987	0.005	0.8	1.000
	PP	20 °C	0.192	0.024	4.2	0.916
		30 °C	0.494	0.002	1.6	0.957
		40 °C	0.987	0.005	0.8	1.000
***S.* Typhimurium**	**Glass**	20 °C	0.195	0.018	4.1	0.890
		30 °C	0.383	0.001	2.0	0.974
		40 °C	0.765	0.002	1.0	1.000
	HDPE	20 °C	0.214	0.087	4.9	0.862
		30 °C	0.383	0.001	2.0	0.972
		40 °C	0.765	0.002	1.0	1.000
	PP	20 °C	0.164	0.008	4.8	0.841
		30 °C	0.383	0.001	2.4	0.998
		40 °C	0.592	0.175	1.8	0.991
***S.* Typhi**	**Glass**	20 °C	0.099	0.008	8.0	0.782
		30 °C	0.233	0.046	3.6	0.919
		40 °C	0.969	0.002	0.8	1.000
	HDPE	20 °C	0.080	0.009	10.0	0.771
		30 °C	0.310	0.005	2.5	0.971
		40 °C	0.369	0.058	2.2	0.951
	PP	20 °C	0.090	0.018	9.6	0.706
		30 °C	0.310	0.005	2.5	0.973
		40 °C	0.744	0.224	1.4	0.967

[a] Linear decline rate constant = (2.303 x Fig. 2 slope gradient). Some R^2 values of 1.000 were generated due to there being only two data points.

4. Discussion

This study has demonstrated that the three most prevalent *Salmonella* serovars that cause of BSI across Africa can readily persist on plastics and glass, under conditions simulating those of urban waste piles that are common in informal settlements. Most importantly, these pathogenic organisms still retain their virulence, confirming that plastic and glass in the environment can act as reservoirs for dangerous clinical pathogens and represent a significant environmental and public health risk.

K-values are often used to inform fate and transfer models of microbial pollutants in soils and water when assessing risks to the wider

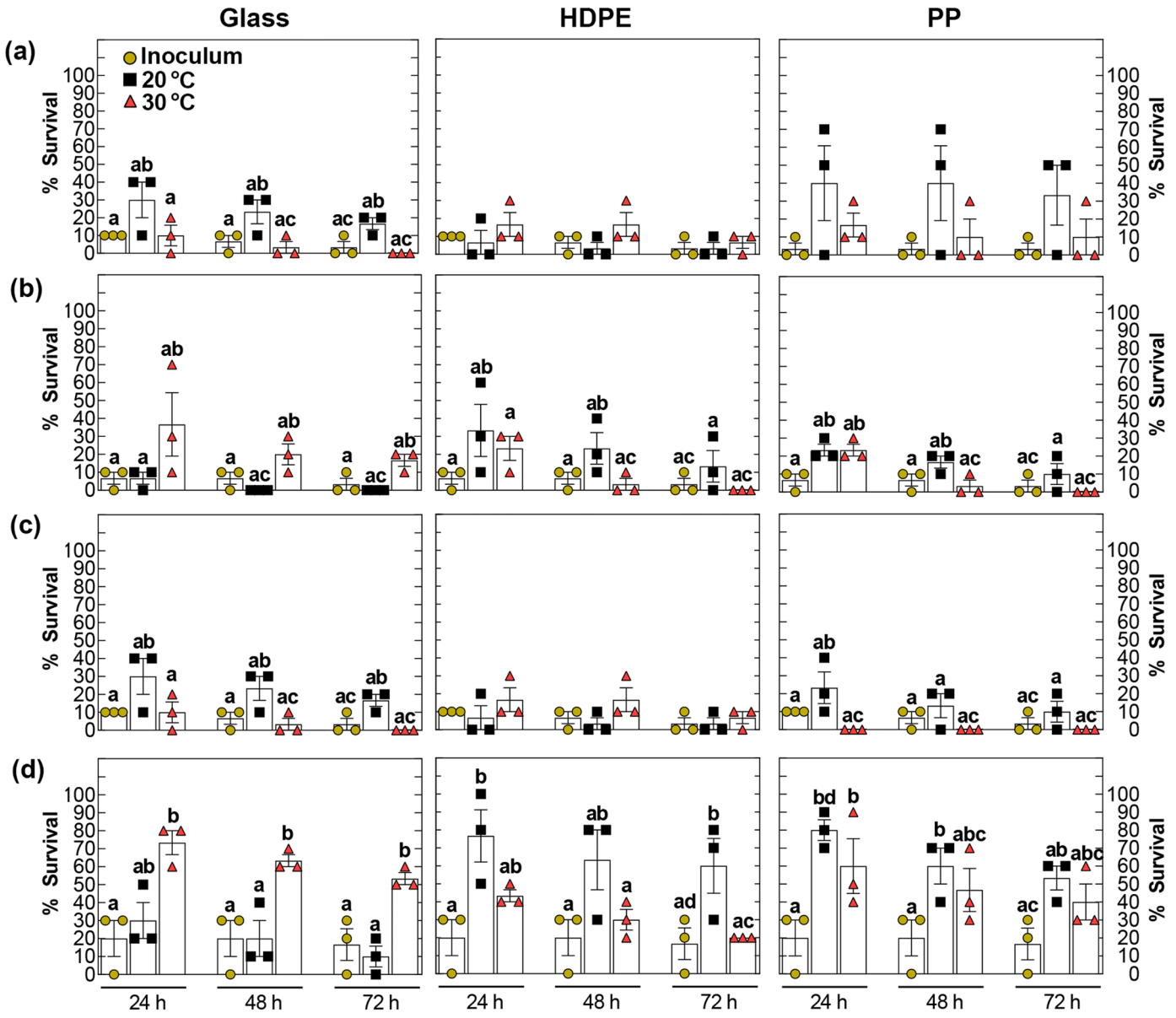

Fig. 3. Virulence of *Salmonella enterica* serovars recovered from glass, HDPE and PP in a *Galleria mellonella* infection model. *Galleria* survival following challenge with *Salmonella enterica* strains (a) *S.* Enteritidis [D7795]; (b) *S.* Enteritidis [CP255]; (c) *S.* Typhimurium [D23580]; and (d) *S.* Typhi [ZH9] recovered from glass, HDPE and PP. Bars with different letters differ significantly from each other (Kruskal Wallis test, with uncorrected Dunn's post-test). Data points represent the mean ($n = 10$ *G. mellonella* larvae) of three independent biological replicates ± SE.

environment but can also determine the likelihood for survival of environmental pathogens in the plastisphere under different conditions. Recent work has revealed the influence of environmental parameters on the die-off rate of pathogenic organisms associated with the plastisphere [35], and on natural materials including sand and in seawater [36,37]. Our study has highlighted the key role that temperature plays in the survival of *Salmonella* spp. within the plastisphere, and with significant interactions occurring between strain, temperature, and material. Different survival rates between strains of *Salmonella* are likely due to population heterogeneity, with subpopulations having different adaptations to environmental conditions and different decay rates.

Temperature is known to play a role in the ability of other bacterial species, including *Vibrio* (*V. parahaemolyticus* and *V. vulnificus*), to form biofilm on glass, HDPE, and PP, with denser biofilms produced at lower temperatures (25 °C) compared to higher temperatures (35 °C), and on plastic compared to glass [38]. While bacteria are known to attach more quickly and to grow and develop biofilms more rapidly on hydrophobic surfaces such as plastics, compared with hydrophilic surfaces, e.g., glass [39], our results demonstrate that *Salmonella* can persist on both materials in the context of an environmental waste pile; however, the structure and composition of the biofilm on both substrates was not quantified.

Human interaction with waste piles will occur mainly at the surface of the pile where temperatures are lower. As the lower temperature facilitated the greatest survival of *Salmonella*, this indicates potential implications for people who directly interact with dump sites in peri-urban communities. Urban waste piles, in LMICs, are composed of many different materials, that are often collected by informal waste pickers to be recycled, resold, or repurposed, although this activity often comes at the cost of high levels of exposure to human pathogens [8]. The lightweight and buoyant properties of plastic can further enhance distribution through the landscape and increase the risk of contact with humans and livestock.

Although the specific reservoirs are unclear, *Salmonella* spp. can

survive for long periods in the environment. It is well documented that animals such as dogs, livestock and birds are attracted to dumpsites in LMICs [7,24], where they often consume human faecal material [25]. Reports have estimated that approx. 9% of human Salmonellosis is caused by direct contact with animals [40], with factors such as food deprivation, or concurrent viral or parasitic disease leading to increased shedding load of *Salmonella* spp. to the environment [41]. The enhanced interactions between humans, animals and the environment (including plastic pollution) could increase the risk of novel diseases emerging and spreading rapidly. As *Salmonella* is the most frequently isolated blood stream pathogen across Africa, identifying a potential direct exposure route for pathogens within communities is vital for eradicating this disease.

The ability of *Salmonella* to persist outside of an animal host is mainly due to its ability to form biofilm, which provides a protective, nutrient rich environment [42,43]. *Salmonella* biofilm formation is influenced by environmental conditions including temperature, pH, glucose, oxygen availability and moisture [44-46]. The warm, humid, and nutrient rich environment of a waste pile provides an exploitative niche for bacterial growth and subsequent survival on the surfaces of materials such as plastics. The temperature during biofilm formation influences the density and stability of the biofilm, as well as any virulence gene expression [47,48]. The production of curli and cellulose, which are major constituents of *Salmonella* biofilm, occurs only at temperatures below 30 °C [49-51]. This suggests that *Salmonella* spp. produce greater amounts of biofilm as a survival mechanism in response to lower environmental temperatures [52]; and it is likely that at temperatures such as 40 °C, the *Salmonella spp.* in our study were unable to persist due to down-regulation of biofilm producing genes. Alternatively, in conditions that more closely mimic host temperatures (e.g., ~40 °C), cells may be primed for dispersal from biofilms. Increases in both rainfall and temperature can increase *Salmonella* abundance and infections [53-55], therefore, at 40 °C, cells may be triggered to transition to a planktonic state rather than a biofilm state.

A recent study, however, has reported that the biofilm forming ability of strains of invasive Nontyphoidal salmonella (iNTS) is likely dependant on the serovar, with certain lineages of African *Salmonella* showing impaired biofilm development [56]. In *in vitro* studies, biofilm formation of *S.* Enteritidis isolate D7795 was shown to be shut off completely; while in *S.* Typhimurium isolate D7795, biofilm formation was significantly impaired. The impairment of the rdar (red, dry and rough) phenotype in the African invasive lineages indicates a distinction from lineages of *S.* Typhimurium and *S.* Enteritidis associated with food supply chains in affluent settings. Here, all strains were capable of producing biofilm under the conditions tested, particularly at lower temperatures, demonstrating that the persistence of each isolate was not associated with differences in their ability to form biofilm. Associating with the plastisphere seems to be enough to provide protection and sustenance and negates the need for strains of *Salmonella* to produce their own environmental biofilm. In the future, studies are needed that examine gene expression within the plastisphere, e.g., using a targeted molecular approach or a broader transcriptomic analysis, to truly reveal the effect of environmental parameters on gene expression within the plastisphere.

In contrast to non-typhoidal strains of *Salmonella*, the environmental reservoir for *S.* Typhi remains elusive, with humans being the only known natural host of S. Typhi. Despite this, infections arise through the consumption of contaminated food or water and subsequent transmission by the faecal-oral route (so-called “long-cycle” transmission); therefore, there must be an ecological niche in which *S.* Typhi can survive prior to human ingested. Although *S.* Typhi has been recovered from environmental sources, there are concerns about the sensitivity and specificity of the molecular approaches used, as DNA from dead bacteria can persist resulting in false identification of bacteria that can cause infection [57]. Recently, culture-based approaches in Malawi have indicated that viable *S.* Typhi can be found in the environment, e.g., in biofilms on the surfaces submerged rocks [58]. Our data indicate that plastics and glass in the environment could also provide a substrate for *S.* Typhi to persist, particularly at the lower temperatures of 20 °C and 30 °C. Some evidence suggests that interaction with eukaryotic species, including species of the protozoan *Acanthamoeba* can enhance survival of *S.* Typhi , *S.* Typhimurium , and *S.* Dublin in the environment and similar interactions could have influenced the persistence seen in our study [59-61]. The eukaryotic component of the plastisphere is relatively understudied [62] but could reveal important insights into how prokaryotic pathogens, such as *S.* Typhi, can survive within mixed community biofilms in the environment.

Salmonella recovered from the plastisphere after 28 days remained lethal in a *G. mellonella* infection model, confirming that abiotic surfaces in waste piles can act as a reservoir for pathogenic *Salmonella* spp., and thus pose a potential threat to human health. Although the infectious dose in humans is unknown for the lineages of *Salmonella* used in this study, the infectious dose of other *S. enterica* strains has been reported to be fewer than 1×10^5 CFU [63,64]. Importantly, the infectious doses may be lower in compromised individuals or if gastric acid production in the gut is reduced, for example, following the use of acid-reducing medications, or if food with a buffering effect (e.g., chocolate) has been consumed, with doses as low as 1 CFU shown to be infectious [65]. *Galleria* larvae are a suitable surrogate for *Salmonella* infection assays, with the infectious dose of *S.* Enteritidis (~10^3 CFU/larvae) [66] and *S.* Typhimurium (~10^2 CFU/larvae) [67] which is similar to that reported for human infection. In this study, all isolates were recovered from glass, HDPE and PP at concentrations greater than 1000 CFU and proved lethal in a *G. mellonella* model. This indicates that *Salmonella* can survive on these materials at concentrations sufficient to cause disease in humans. Following recovery from the plastisphere, most isolates showed an enhanced pathogenic potential with the induced lethality in the *Galleria* model being greater than the expected mortality at specific concentrations. It has previously been suggested that the plastisphere may select for virulence [16]; as such, we hypothesise that the nutrient levels found within the plastisphere may increase pathogen virulence by favouring transmission of fast-growing virulent strains, or by allowing pre-adaptation to efficient use of resources following dissemination to a nutrient rich host.

While persistence at lower temperatures was likely due to enhanced biofilm formation, the concurrent retention of virulence is likely due to virulence gene co-regulation. *Salmonella* biofilm and virulence genes are often co-regulated in response to Quorum sensing (QS), which is a cell-cell communication mechanism dependent on cell density that co-ordinates bacterial group behaviours such as bioluminescence, biofilm formation, and virulence factor production [68,69]. QS is dependent on the production, release, and detection of signalling molecules called autoinducers [70], which regulate a variety of bacterial phenotypes, including biofilm formation, conjugation, motility and virulence gene expression. In *S.* Enteritidis for example, autoinducers can simultaneously increase the expression of biofilm formation genes (*lpfA, fimF, fliF, glassgC*) and virulence genes (*hilA, invA, invF*) [71]. The role of the plastisphere in autoinducer expression has not yet been examined, although substrate surface could potentially be involved with the induction of genes for biofilm formation and virulence.

For most of the *Salmonella* isolates used in this study, material type and temperature did not affect virulence in the *Galleria* infection model, *S.* Typhi isolate ZH9 did appear to have reduced virulence capabilities when recovered from glass at 30 °C (relative to the initial inoculum); and from HDPE and PP at 20 °C (relative to the initial inoculum). *S.* Typhi strain ZH9 is an attenuated strain, derived from isolate Ty2 which harbours deletion mutations in the *aroC* (encodes chorismate synthase, an enzyme involved in the biosynthesis of aromatic compounds) and *ssaV* (encodes a component of the type III secretion system encoded on *Salmonella* pathogenicity island 2 [SPI-2]) genes [31]. The roles of SPI-2 in both virulence and biofilm formation are well described [72,73], and it could be hypothesised, therefore, that the reduced virulence and

persistence of *S.* Typhi isolate ZH9 in this study were due to this. The importance of SPI-2 in the environmental survival of *S.* Typhi, and indeed all *Salmonella* strains, should be examined in future studies.

5. Conclusions

Substrates commonly found in urban waste piles can provide an important ecological reservoir for different species of *Salmonella* to survive, and importantly, retain their virulence. In the environment, plastics can persist for decades or even centuries [74], and thus provide increased opportunities for colonisation (and subsequent re-colonisation) by pathogens from clinical and environmental contamination. Wildlife and livestock interacting with waste piles represent an additional risk to humans through zoonotic transmission. This study has demonstrated that communities in sub-Saharan Africa interacting with waste piles to dispose of waste, or to recover and repurpose items, are therefore at risk of interaction with dangerous human pathogens. However, plastic pollution is a global issue, and the implications of human pathogens colonising environmental plastic waste demonstrates a potentially widespread public health issue that needs significant investment in terms of resources and research in order to inform future policy and health legislation relating to plastics in the environment.

Environmental implication

Plastics that become colonised by pathogens constitute significant environmental and human health implications. The lightweight and buoyant properties of plastic allow for enhanced distribution through the landscape, which can result in increased risk of contaminating areas where direct human exposure can be high. Our data indicate that human pathogens can survive, and most importantly can retain their virulence, on waste plastic and glass for at least 28-days, at temperatures associated with the surface of peri-urban waste piles. This demonstrates that plastic pollution heightens the co-pollutant risk of plastic pollution, and the increased risk of humans being exposed to dangerous clinical pathogens in the environment.

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CRediT authorship contribution statement

MJO, **RSQ**: Conceptualisation; **MJO**, **HW**, **RM**: Data curation; **MJO**: Formal Analysis; **MJO**: Writing-Original draft preparation; **RSQ**: Supervision; **MJO**, **HW**, **RM**, **DMO**, **NAF RSQ**: Writing, reviewing, and editing; **RSQ**: Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.132439.

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