Effect of Soil Type and Temperature on Survival of *Salmonella enterica* in Poultry Manure-Amended Soils

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/lam.13302

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Significance and Impact of the Study

The persistence of *Salmonella enterica* in soil environments was shown to be significantly influenced by a range of individual and interacting environmental effects, including temperature, soil type and amendment addition. This indicates that current horticultural food safety management systems which employ a uniform prescribed exclusion period between application of manure and time of harvest may be unfit for purpose under certain conditions by either underestimating or overestimating pathogen die-off. These findings support exclusion periods that account for a range of environmental factors including temperature, soil type and growing region may be more appropriate to manage microbiological risks associated with soil which has been amended with manure.

Abstract

The effects of soil type and temperature on the survival of a cocktail of five *Salmonella enterica* serotypes (Enteritidis, Infantis, Montevideo, Typhimurium and Zanzibar) in manure-amended soils under controlled laboratory conditions was assessed. Containers of clay loam or sandy soil, unaltered or amended with 2% (w/w) poultry manure, were inoculated with *S. enterica* (~5 log10 CFU g-1) and held at 5, 21 or 37°C for 6 weeks. Statistical analysis of the persistence of *S. enterica* identified a significant three-way interaction between soil type, manure amendment and temperature. Clay loam soils and lower temperatures tended to support *S. enterica* persistence over six weeks with only 1-log and 2-log reductions, respectively. In contrast, sand and higher temperatures resulted in a 4-log and either 3-4-log reductions, respectively. Manure amendment had an overarching effect of reducing die-off of *S. enterica* in comparison with unamended soils. This study highlights that a large component of variation of the rate of *S. enterica* reduction in soils may be attributed to combinations of environmental factors, in particular, soil type and temperature. It further underscores the importance of risk management strategies and industry guidelines based on local data and that reflect the diversity of prevailing horticultural production environments.

Keywords: Soil remediation, horticultural food safety, *Salmonella* spp., poultry manure, die-off

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Introduction

Diarrheal and invasive non-typhoidal *Salmonella enterica* are amongst the foremost contributor to the global burden of foodborne disease (Kirk *et al*., 2015). In Australia, salmonellosis is the second leading cause of notified foodborne illness (Ford *et al.*, 2016) and, of the major gastroenteric pathogens, *Salmonella* spp. incur the greatest disease burden in terms of acute gastroenteritis-related fatalities and average severity of disease. Fruits and vegetables in general are increasingly recognized as an important vehicle for the transport of human pathogens that were previously assumed to be associated with foods of animal origin (Berger *et al*., 2010). Recent years have seen an increase in reported cases of salmonellosis and outbreaks associated with fresh produce (Bennett *et al.*, 2018; Ford *et al*, 2016; Gibbs, 2009; Munnoch, 2009). The increase in salmonellosis cases associated with fresh produce may be influenced by factors such as an increase in consumption of fresh produce, a lack of effective decontamination methods for produce, intensification of livestock production, supply chain complexity, and increasing numbers of immunosuppressed consumers (Olaimat, 2012; Strawn *et al*., 2013; Wadamori *et al*., 2017).

There are multiple factors that could result in produce contamination on-farm. Irrigation water, manure, run-off and animal intrusion have all been noted as potential routes of transmission for *Salmonella* spp. on farms (Harris *et al.*, 2003; Gerba and Smith, 2005; Hanning *et al.*, 2009; Lynch *et al.*, 2009). The ubiquitous and adaptable nature of *Salmonella* spp. aids in its ability to persist in farm environments (Kupriyanov *et al.*, 2010; Jacobsen and Bech, 2012). It has been demonstrated that *Salmonella* spp. and *Escherichia coli* have the ability to cycle throughout the on-farm environment moving from soil to animal to waste to soil (Kupriyanov *et al.*, 2010).

Aside from animals and water as reservoirs, pathogens can also be unintentionally introduced into crop field soils through amendments such as treated manures and biosolids as fertilizers (Gerba and Smith, 2005). Under typical cropping conditions pathogens may be protected from environmental conditions that would otherwise result in rapid die-off. This can result in their survival for weeks or months in agricultural environments. For example, Islam *et al.* (2004) demonstrated that *S.* Typhimurium persisted for up to 231 days in soils amended with contaminated manures, even after plants had been harvested or died out. They suggested that coverage of soil by lettuce and parsley contributed to the prolonged survival of *S.* Typhimurium by physically protecting the bacteria from variations in weather conditions (Islam *et al.*, 2004). In a similar study, inoculated *S.* Newport took 107 days to decline from 10^7 CFU g^{-1} to the limit of detection in non-sterilized soils amended with manure. When the soil was sterilized before treatment and inoculation, removing competitive microflora, *S.* Newport was detectable for 158 days (You *et al.*, 2006).

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The prolonged survival of *S .enterica* and other pathogens in manure-amended soils is of particular concern in fields with crops that undergo minimal processing prior to consumption, such as leafy green vegetables. The transfer of pathogens from soil to plants may occur through either direct contamination from the soil or potentially surrounding water sources through run-off or leaching (Bech *et al*., 2009; Jacobson and Bech, 2012; Ongeng *et al*., 2011). Exclusion periods for harvest are commonly employed to mitigate against risks associated with amendment of soils by raw manures. The National Organic Program of the United States Department of Agriculture currently requires a waiting period of either 90 or 120 days between application of raw manure and harvest of plants with edible parts (USDA, 2000). The length of the waiting period is not standardized world-wide, with horticulture production systems in other counties employing both shorter and longer periods. In Australia, the current industry guidelines for fresh produce stipulate an exclusion period from the time of application of fertilizer or soil additive to the time of harvest (Fresh Produce Safety Centre Australia & New Zealand, 2019). For fresh produce that is not treated with an approved treatment process and may be eaten raw, the exclusion period is 90 days when the harvestable part is grown in direct contact or close proximity to soil, and 45 days when it is not (Freshcare, 2016).

The effectiveness of an exclusion period is based on an assumption that an appropriate amount of time has passed to mitigate against the risks of enteric pathogens, including *Salmonella* spp., in amended soil. However, it should also be recognised that there is significant regional variation in environmental, microbial diversity and production practices. As such, it is necessary to elucidate how environmental factors that can vary amongst growing regions may contribute to persistence of regionally relevant (in this case Australian) poultry isolates of *Salmonella* spp. in the soil environment. The *in vitro* study presented aimed to assess the interaction of environmental factors soil type, temperature, and with poultry manure amendment, impacting *Salmonella* spp. survival over 6 weeks, representing the minimum length of time for the exclusion period between application and harvest for low risk produce in Australia (Freshcare, 2016). An understanding of interactions between these key environmental factors will provide information critical for improving on-farm risk management for fresh produce.

Results and Discussion

Our results confirmed that *S. enterica* survival was significantly influenced by complex environmental interactions involving soil type, manure amendment and temperature. Differing non-log-linear declines were observed, which prevented direct comparison using conventional predictive log-linear and non-log-linear modelling methods, however a number of trends were observed. When comparing the main effects, pathogen survival was enhanced in clay loam compared to sand (Figure 1), at lower temperatures (Figure 2) and in

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manure-amended soils compared to unamended soils (Figures 1 and 2). All of the main effects and two-way interactions, and several of the three-way interactions tested in this study were shown to significantly affect *S. enterica* survival.

S. enterica was found to die-off more rapidly in sand than in clay loam (Figure 1). In comparison to sand where *Salmonella* approached the limit of detection after 6 weeks, *S. enterica* in clay soils was shown to persist throughout the entire duration of the study. Distinct differences in the shape of survival curves were observed between soils. This was most clearly observed in clay loam soils where tailing and/or increases in levels of *S. enterica* were observed after 4 weeks.

Soil type is generally considered to be an important factor affecting the survival of enteric pathogens. For example, higher clay content has previously been shown to improve pathogen persistence of *S. enterica* and *E. coli* (Wang *et al.,*2018) and other pathogenic bacteria (Awasthi *et al*., 2019). However, the relationship between soil texture (as defined by sand:silt:clay composition) and pathogen survival is not always consistent due to the influence of other factors and interactions such as pH, electrical conductivity and moisture content (Erickson *et al*., 2014). For example, the persistence of *E. coli* O157:H7 and *S.* Typhimurium in sandy and loamy soils can be enhanced by manure derived from a high fibre cattle feeding regime, and its high pH (Franz *et al*., 2005). How clay content influences bacterial survival is not fully understood, but may be associated with increased buffering capacity, moisture retention and nutrient availability; and smaller particle sizes that offer protection from predators, parasites and desiccation, and exposure to ultraviolet light and toxins (Brennan *et al*., 2014). Clay minerals have been shown to alter the physicochemical properties of soil via multiple mechanisms, including cation exchange capacity and surface area, both of which have a differential impact on bacterial survival (Brennan *et al*., 2014). Given this it is likely that growing regions with clay soil environments may require longer remediation periods than sandy environments in manure growing environments which have had amended with uncomposted manures.

Incorporation of manure into soil was shown to support the persistence of inoculated *S.enterica* in comparison to unamended soil (Figure 1). In unamended soils *S. enterica* declined 2-log over 4 weeks while in manure-amended soils the decline was 1-log over the same period, however after a 6-week period had returned to near original concentrations . Poultry manure and litter are widely used in agricultural production due to their high nutrient content and relatively low cost (Bolan *et al.*, 2014). Composting is generally thought to be an effective method for eliminating food-borne pathogens in animal manures, however there have been a number of studies reporting the persistence of pathogens during composting (see reviews by Gertler *et al*., 2018; Sharma *et al*., 2018) and, on many farms, uncomposted or minimally managed manures may be used. In addition to the

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potential to introduce pathogens, animal manures may also support pathogen survival by altering soil characteristics including water holding capacity (WHC), nutrient availability, pH, organic carbon and electrical conductivity (Antil and Singh, 2007, Dhaliwal *et al*., 2019).

A general trend towards increased die-off with increasing temperature in unamended soils (Figure 2 amd Table 2), the time to achieve a 2-log reduction based on an interaction model at 5, 21 and 37°C were 6, 2 and 1 weeks respectively. Enhanced persistence of *S. enterica* was found for manure-amended soils compared to unamended soils at 21 and 37°C with all temperatures requiring at >5 weeks. Observed reductions at higher temperatures were greater in unamended soil than amended soil suggesting that manure provided a source of nutrients promoting survival of enteric pathogens such as *Salmonella* spp. (Sharma et al., 2016). The prolonged survival of *Salmonella* spp. at cooler temperatures in amended and non-amended soils, compared to warmer temperatures may be associated with cold stress response in *Salmonella* spp. (Ricke *et al*., 2018). A cold stress response may help protect bacteria from secondary stresses (Xu *et al*., 2008), and may be responsible for no differences (in weeks required for 1- or 2- log reduction) in amended or unamended soils held at 5°C. Further studies characterising the stress response of *Salmonella* spp. in soil environments may further elucidate the mechanisms of this putative persistence.

A significant environmental variable that was excluded from this experiment was moisture. Many studies have indicated that the moisture status of soil is an important factor affecting the survival of enteric pathogens (Holley *et al.,* 2006; Lang and Smith, 2007) such that limited moisture availability in soil reduces survival rates of enteric bacterial pathogens in soil or manure-amended soil systems (Jamieson *et al.* 2002). In a survival study in a soil system held at 25°C, both *E. coli* O157:H7 and *Salmonella* spp. was affected by moisture content with greater losses in soils adjusted to 40% water holding capacity (WHC) compared to soils adjusted to 20% WHC (Erickson *et al.* 2014). It was also reported that drying reduces the number of *S.* Typhimurium found in manure and litter, however, drying is only effective at certain levels of water activity. When most of the water has been removed, *Salmonella* spp. will survive for longer periods of time (Himathongkham and Riemann 1999). Further research on the interaction of both soil and manure moisture content on *S. enterica* persistence may provide further insights.

The observation of non-log-linear reductions and even growth in some cases was of particular note, particularly as exclusion guidelines and regulations are based on an assumption of a reproducible and consistent decline. Similar non-log-linear declines have been encountered in in field studies (Wang *et al*., 2018). Whether this may represent alteration of the metabolic functions in the inoculated cells due to equilibration within pot environment or perhaps the resuscitation of viable but non-culturable cells is unclear. To further support the

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validation of evidence-based regulatory controls, the development of robust mechanistic models to adequately account for the non-linear reductions are a critical need.

Implications of this research for on-farm guidelines, in particular, the efficacy of an exclusion time for pathogen reduction treatments, were assessed by observing the weeks to achieve 90% (1-log CFU g^{-1}) and 99% (2-log CFU g^{-1}) reduction in *S. enterica* population. The time required to result in a 1-log reduction was at least twice as long in unamended clay loam compared to unamended sand; however manure amendment extended the log reduction time by 1–2 weeks in both soil types (Table 2). The time to achieve a log reduction in unamended soils at 5°C was approximately 3 weeks compared to 1 week at 21 or 37°C; however the addition of manure amendment resulted in similar log reductions across all temperatures (Table 2).

In conclusion, it was found that environmental factors interact significantly in their effects on pathogen survival, with substantial differences in log reduction times due to soil type or manure amendment. In particular, sandy soils promoted the die-off of *S. enterica* at a much higher level than for clay soils. The addition of manure and lower temperatures further promoted persistence of *S. enterica.* These findings support the use of on-farm food safety guidelines but suggest that the exclusion period of 45 days for low risk crops, designed to reduce *S. enterica* counts by 1-2 log, is not uniform across different environments. Our data suggest they need to be finessed according to region and localized studies that take into consideration the diverse environmental conditions of resident production systems.

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Materials and Methods

Experimental design The study was a full-factorial design with three factors: soil type (sand or clay loam), manure amendment (0 or 2% w/w poultry manure in soil), and temperature (5, 21 or 37°C). Three replicates for each treatment combination were destructively sampled each week for a period of 6 weeks. Uninoculated control samples were assessed at the start and end of the experiment to check for unintentional contamination.

Sample preparation Bulk soil (approximately 100 kg) was collected from two research properties owned by the University of Sydney. Sand was collected from Karalee Farm (Camden, New South Wales, Australia) located approximately 70 km from Sydney (34°00'57.90" S, 150°40'19.17" E). Soil (0–10 cm depth) was taken from an area previously used for pasture and small-scale horticultural crop production. Clay soil was collected from John Bruce Pye Farm (Camden, New South Wales, Australia) located approximately 60 km from Sydney (33°56'33.92" S, 150°40'06.02" E). Soil (0–10 cm depth) was taken from an area dominated by Red Sodosol (Commonwealth Scientific and Industrial Research Organisation, 2016) and used for large-scale cereal breeding trials. Key physicochemical properties of the soil samples are shown in Table 1.

Soil samples were air-dried, mixed thoroughly, and sieved to 2 mm before use in experiments. The experimental units were 120-mL screw-top containers containing 100 g sand or clay loam. The manure-amended treatments contained 2% (w/w) friable poultry manure, which was purchased from a commercial supplier as an aged product (stored in large outdoor piles for up to 6 months with no additives).

***Salmonella* strains** Five *Salmonella enterica* serovars were used in this study, *S.* Enteritidis, *S.* Infantis, *S.* Montevideo, *S.* Typhimurium and *S.* Zanzibar. The cultures were obtained from Birling Avian Laboratories (New South Wales, Australia) and maintained on tryptic soy agar (TSA) plates. Isolates were selected as all had originally recovered Australian poultry flocks. The purity of isolates was ensured by re-isolating from overnight cultures (37°C, 18–24 h) on xylose lysine deoxycholate agar (XLD; Becton, Dickinson and Company, USA), sub-culturing to nutrient agar (NA; Becton, Dickinson and Company, USA), then confirming the serogroup of single colonies using somatic antisera (Staten Serum Institute, Denmark) in slide agglutination tests.

Inoculation Lawn cultures of each *S. enterica* serovar were prepared on TSA plates, transferred to phosphate buffer (pH 7.2), diluted, and mixed together to produce ~6.5 $\log_{10}$ CFU mL^{-1}liquid inoculum based on optical density with each serovar present in approximately equal proportions. Each sample container was inoculated with 5 mL inoculum to achieve an initial *S. enterica* target concentration of ~5.0 $\log_{10}$ CFU g^{-1} soil. An additional 5 mL sterile water was added to each sample to result in a moisture content of 15% (w/w) in the

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sand and 20% (w/w) in the clay loam. The containers were thoroughly mixed by rotation on a drum-mixer for 20 min and incubated with screw-caps firmly closed to prevent moisture loss at either 5, 21 or 37°C.

Recovery and Enumeration Inoculated and uninoculated soil samples were extracted according to (Lopez-Velasco, 2012), with slight modifications. Each soil sample (100 g) was weighed into a sterile 400 mL filter bag (Interscience, France) and manually massaged for 1 min in 100 mL soil extraction medium (0.02 M sodium phosphate with 0.1% Tween 20). After allowing the suspension to settle for 15 min, 50 µL aliquots of the supernatant were spread onto duplicate XLD (Difco, USA) agar plates using an automatic spiral plater (easySpiral, Interscience, France). The plates were incubated overnight (37°C, 18–24 h) and then enumerated using an automatic colony counter (Scan 500, Interscience, France). Colonies with typical morphology (i.e., red colonies with black centres) were presumptively identified as *S. enterica*. Random plates were intermittently selected and 5-10 colonies checked with antisera as previously described. If no colonies were recovered the samples were enriched in tetrathionate broth (TTB, Becton, Dickinson and Company, USA) and plated onto XLT4 (Becton, Dickinson and Company, USA).

Data analysis *S. enterica* survival was analyzed statistically in R using a generalized least squares model (nlme package) weighted by time to account for non-constant variance in time. Three-way interactions between soil, manure amendment, temperature and time were visualized using plots of estimated means and 95% confidence levels generated using the effects package (Fox, 2003).

Acknowledgments

This research was funded by Horticulture Innovation Australia (project number VG13039) using the Vegetable Industry Levy, co-investment from the University of Sydney, and the Australian Government. This research was also supported by the Center for Produce Safety, United States.

Conflict of interest

No conflict of interest declared

Supporting Information

Table S1. Mean log reductions of *S. enterica* per weekly interval.

Figure S1. Interaction plot of soil, manure and temperature interactions on *Salmonella* survival.

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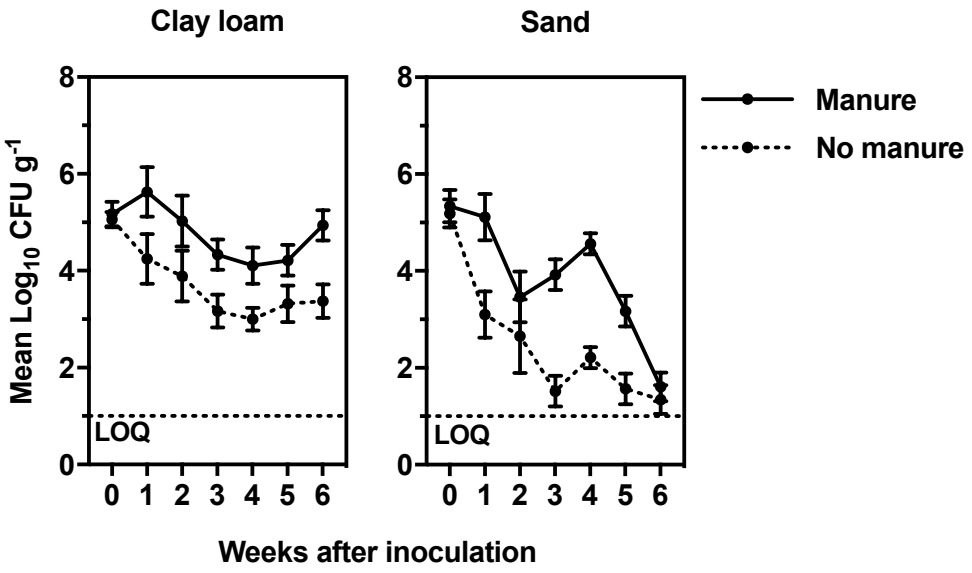

Figure 1. Effects of soil and manure on *S. enterica* survival over a 6-week period. Points represent pooled mean values across all temperatures and error bars indicate 95% confidence levels.

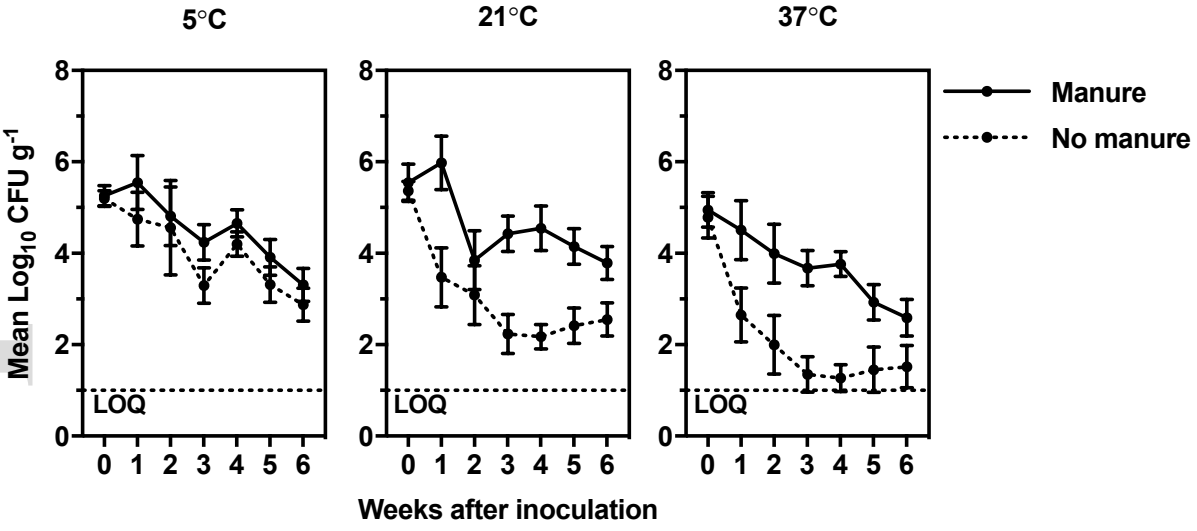

Figure 2. Effects of temperature and manure on *S. enterica* survival over 6-week period. Points represent pooled mean values across all soil types and error bars indicate 95% confidence levels.

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Table 1. Physicochemical properties of the two soil types used in this study.

	Soil parameter[b]						
Soil sample[a]	Bulk density (g cm^{-3})	Total C (%)	Total N (%)	Ammonium (ppm)	Nitrate (ppm)	pH	EC (µS)
Clay loam	1.1	4.24	0.38	7.7	15.0	5.06	512.40
+ manure	-	4.52	0.44	10.0	14.7	6.19	907.20
Sand	1.3	0.72	0.05	1.2	2.5	5.41	85.38
+ manure	-	1.10	0.11	5.3	2.3	7.53	592.60

[a] manure amendment with 2% (w/w) poultry manure; [b] values are the average of five replicate analyses.

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Table 2. Minimum time required to achieve a 1- or 2-log reduction in *S. enterica* population from an initial inoculum load of ~5 $\log_{10}$ CFU g^{-1} based on a model of the soil-manure-time interaction (n=3).

Main Effects	**Manure**	**Weeks to achieve a reduction of**	
		1-log	2-log
Soil Type			
Clay loam	-	2	4
	+	4	>6
Sand	-	1	1
	+	2	5
Temperature			
5°C	-	3	6
	+	3	6
21°C	-	1	2
	+	3	>6
37°C	-	1	1

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