**Title:** The persistence of *Salmonella* following desiccation under feed processing environmental conditions: a subject of relevance.

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Running title: Active but non-culturable *Salmonella*

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**Significance and Impact of Study:**

While *Salmonella* has been shown to persist for years in feed processing environments, it is still unknown how temperature and humidity affect the persistence of *Salmonella* cells over time in terms of their metabolic states and cultivability. Here we show that long term exposure to feed processing environmental conditions induces *Salmonella* into a non-culturable state even though about 1 % of the population remains metabolically active. This has significant implications when monitoring *Salmonella* from the environment which could yield false negative results using conventional pre-enrichment detection methods.

**ABSTRACT**

Although *Salmonella* persistence has been predominantly linked to biofilm formation, the physiologic state of *Salmonella* should also be considered as a possible pathway for persistence and survival in feed the industry. Hence, the purpose of this study was to assess the extent of viability of *Salmonella* cells through long-term desiccation periods under conditions typically found in feed processing environments, and whether these same cells could resuscitate and cause salmonellosis *in vivo.*

We showed that desiccation of *Salmonella* Agona, a representative feed industry isolate and *Salmonella* Typhimurium ATCC 14028*,* a laboratory strain, were induced into a non-culturable state at 35% and 85% relative humidity conditions, at defined temperatures of 30°C and 12°C respectively. Although the reduction in culturable cells was more than 6 log10, metabolic activity was found in more than 1% of the population. Desiccation-induced non-culturable *Salm.* Typhimurium could not be revived and were non-virulent in a mouse model following infection through oral gavage. These results suggest that the specific conditions for reviving non-culturable *Salmonella* after long periods of desiccation are yet to be fully identified. The need for mapping key factors involved in the persistence of *Salmonella*, would help better detect it and improve feed safety measures.

**KEYWORDS***: Salmonella*, Active But Non-Culturable, survival, desiccation, virulence

**INTRODUCTION**

The presence of *Salmonella* on feed processing surfaces may lead to contamination of feed products, increasing the risk of foodborne infections in animals and humans alike ([Crump *et al.* 2002](#_ENREF_5)). *Salmonella* serovars, including *Salmonella* Agona, *Salmonella* Montevideo and *Salmonella* Senftenberg are known to survive and persist in feed processing environments for years ([Nesse *et al.* 2003](#_ENREF_18)). Consequently, feed factories usually implement strict control measures, based on bacteriological monitoring of production environment and products. Samples from the environmental monitoring program are inoculated in pre-enrichment solution to allow growth before detection by selective media or PCR-based methods; methods solely based on detecting the presence of culturable cells. It has been previously shown that *Salmonella* cells may enter a non-culturable physiological state in which they still remain metabolically active and possess the ability to resuscitate ([Oliver 1993](#_ENREF_20)[Oliver 2000](#_ENREF_21)). Lack of multiplication of *Salmonella* in the pre-enrichment medium, either by sub-lethal injury or a general non-culturable state will increase the detection limit of the monitoring methods to a high level. Whether this non-culturable state poses a real health hazard is dependent on the ability of the cells to cause illness or resuscitate. In principal, non-culturable and metabolically active cells capable of regaining their ability to multiply and cause infections are defined as Viable-But-Non-Culturable (VBNC), whereas non-culturable and metabolically active cells that fail to revert back into a culturable state, may only be referred as Active-But-Not-Culturable (ABNC)([Dinu *et al.* 2009](#_ENREF_7)). It is therefore important to distinguish between VBNC and ABNC as the latter cannot be regarded as a risk and should preferably not be detected as viable *Salmonella* in a monitoring program.

A non-culturable state can be induced by stress factors such as sub-optimal temperature ([Cook and Bolster 2007](#_ENREF_4)), osmotic stress ([Asakura *et al.* 2008](#_ENREF_2)), or nutritional starvation ([Arana *et al.* 2004](#_ENREF_1)). Feed processing environments are known for varying temperatures and humidities and could therefore potentially induce a VBNC-state in *Salmonella*. It has been demonstrated that VBNC-cells may resuscitate and grow on nutrient agar after exposure to heat shock, antioxidants, H2O2-degrading compounds and incubation in anaerobic environments or in nutrient-rich medium ([Reissbrodt *et al.* 2002](#_ENREF_26)[Gupte *et al.* 2003](#_ENREF_10)). Interestingly, the presence of synthesized and specific molecules secreted by culturable cells has been shown to revert the viable but non-culturable cells to culturable cells. In one study, resuscitation factor secreted by *Salmmonella* TyphimuriumLT2 cells was shown to successfully revive VBNC *Salmonella* Oranienburg cells into a cultivable state ([Panutdaporn *et al.* 2006](#_ENREF_22)). This observation was used to explain why VBNC *Salm.* Oranienburg cells were able to resuscitate following passage in mice in an earlier study ([Asakura *et al.* 2002](#_ENREF_3)). Non-culturable *Vibrio cholerae* and *Vibrio vulnificus* have also been reported to recover in rabbit and mouse intestines, respectively ([Porter and Feig 1980](#_ENREF_25)[Rodrigues *et al.* 1992](#_ENREF_27)). Dhiaf and Bakhrouf found that resuscitation of VBNC *Salmonella* cells on evaporated sea water salt crystalsoccurred when the bacteria were directly administered orally into the mice, but not if they were administered by intra-peritoneal injection, indicating that the intestinal environment might be essential for resuscitation ([Dhiaf and Bakhrouf 2008](#_ENREF_6)). In other experiments, non-culturable *Salmonella* cells were unable to cause infections in chicken or mice, indicating that cells failed to resuscitate during passage through the gastrointestinal tract ([Lesn *et al.* 2000](#_ENREF_16)[Smith *et al.* 2002](#_ENREF_29)). Why some non-culturable cells are capable of resuscitating and others not, could possibly be dependent on how they were originally induced into a non-culturable state. The non-culturable-state is regarded as an intrinsic strategic survival mechanism, allowing persistence amidst adverse environmental conditions ([Roszak and Colwell 1987](#_ENREF_28)[Nystrom 2003](#_ENREF_19)). Defining the VBNC-state has over the years generated substantial debate among microbiologists and food/feed safety authorities, partly due to contradicting reports on non-culturable cells’ return into a culturable state ([Kell *et al.* 1998](#_ENREF_15)[Pinto *et al.* 2011](#_ENREF_24)) or the VBNC virulence or loss thereof ([Smith *et al.* 2002](#_ENREF_29)). Granting that *Salmonella* has been shown to persist for years in feed processing environments, it is still unknown how temperature and humidity affect the persistence of *Salmonella* over time in terms of their metabolic states and cultivability. Hence, the purpose of this study was to first determine whether a representative feed industry isolate of *Salm.* Agona and the reference strain *Salm.* Typhimurium ATCC 14028 could be induced into a non-culturable state through desiccation under feed processing environmental conditions, and if so, whether the bacteria could be resuscitated and cause salmonellosis *in vivo.*

**RESULTS AND DISCUSSION**

**Activity - and culturability of desiccated *Salmonella* cells**

More than 6 log10 reductions in number of culturable cells were found after respectively 9 and 21 days of incubation of *Salm.* Agona at 85 % and 35 % RH (Figure 1). The results were in accordance with other studies on the survival ability of *Salmonella* under dry environmental conditions ([Pedersen *et al.* 2008](#_ENREF_23)[Iibuchi *et al.* 2010](#_ENREF_13)). The survival of *Salmonella* after desiccation as measured by cultivability on agar plates is dependent on the relative humidity in the surrounding air, and lower humidity is not necessarily associated with lower survival ([Habimana *et al.* 2010](#_ENREF_11)). After 30 days, 6.5 and 7.0 log10 reductions of culturable *Salm.* Agona cells were observed following desiccation at 35 and 85 % RH, respectively. No culturable *Salm.* TyphimuriumATCC 14028 cells were detected after 30 days desiccation at 35 and 85 % RH (Figure 2AB). About 6 log10 cells/coupon were still metabolically active, showing esterase activity (hydrolysation of carboxyfluoresein diacetate), cell membrane pump efflux functionality (efflux of fluorescein in presence of glucose), as well as cell respiratory activity (CTC hydrolysation) (Figure 2AB). The results indicated that about 1 % of the *Salmonella*-population entered into a metabolically active but non-culturable state after subjected to a desiccation process. In a similar study it was found that *Salm.* Typhimurium,exposed to a combination of extremely low humidity (3.5 % RH) and starvation condition, showed signs of esterase activity despite having lost cultivability ([Lesn *et al.* 2000](#_ENREF_16)). When comparing the morphological state of *Salm.* Agona cells following 1 h desiccation (Fig 3A) and those after long exposure periods, scanning electron microscopy revealed that 2-month desiccation at 35% RH led to what appeared to be a film-like substance partially covering some cells (Fig 3B). Cell morphological characteristics seemed unaffected by the long desiccation under 35 % RH compared to morphological state of *Salm.* Agona cells after 1 h desiccation. Compared to 1 h or 2 months desiccation at 35 % RH, *Salm.* Agona cells subjected to 2 months exposure to semi-humid conditions (85 % RH) showed increased signs of long tubular structures connecting cells (Fig 3C), as well as an increased presence of a granular-like substance entirely covering cells. Alteration in cellular morphology was also evident even though majority of cells were somewhat hidden under the covering substance. These observed physical attributes associated with desiccation could indicate the presence of both cell communication and adaptive response to environmental stress conditions. Further transcriptomic analyses of desiccation-induced VBNC/ABNC cells would help better characterize the physiological and adaptive nature of these cells. This can now be made possible through the combination of cell sorting flow cytometry technologies with classical microarray analysis. Such studies would help shed light on whether VBNC/ABNC cells are indeed actively communicating during environmental stress fluctuations, through the use of tubular structures as recently demonstrated. ([Dubey and Ben-Yehuda 2011](#_ENREF_8)). The observed granular-like substance covering the cells at 85 % RH could have been the result of such nanotube communication, activating a cascade of regulatory genes, leading up to the synthesis of a protective slimy layer. A previous study conducted on *Salmonella* has identified an O-antigen (O-Ag) capsule allowing the mediation of protective effects against desiccation ([Gibson *et al.* 2006](#_ENREF_9)). The synthesis of O-Ag at different environmental conditions should be subject of further investigation, which would help explain the morphological differences observed for cells subjected to dry (35 % RH) and humid (85 %) environmental conditions. This carries consequences for cross–contamination and feed safety since O-Ag was shown to be a contributing factor for the survival of *Salmonella*. sp in desiccated foodstuffs ([Hiramatsu *et al.* 2005](#_ENREF_12)).

***Virulence and resuscitation of non-culturable cells***

To investigate whether non-culturable *Salm.* Typhimuriumcells (incubated at 85 % RH for 35 days) still could be virulent in an *in vivo* environment, a mouse model with BALB/c mice was used. This is a widely used model where virulent activity is easily detected due to the fact that *Salm.* Typhimuriumcauses a systemic infection. In our experiment, we also infected groups of mice with different doses of culturable bacteria of the same strain to observe the level of virulence and the relevant symptoms of salmonellosis of this strain. The range of onset of clinical symptoms was from day three to day seven. *Salmonella* was isolated from liver/spleen, intestinal tract and blood from all animals that displayed clinical symptoms. *Salmonella* was not isolated from any of the clinically healthy animals, indicating that sub-clinical infections did not occur. Of the animals infected with culturable *Salmonella*, all three given 108 cfu, three of the six given 106 cfu and one of the six given 104 cfu developed salmonellosis, i.e. systemic infection and clinical symptoms (Table 1). On the other hand, all mice infected with 108 non-culturable bacterial cells remained symptomless throughout the observation period, and *Salmonella* was not isolated from any of the samples from these animals.

The ability to resuscitate after entering a non-culturable state after desiccation was also tested *in vitro* with several methods, such as temperature shock, addition of supernatant of a *Salmonella* overnight-culture and anaerobic incubation, but *Salmonella* failed to grow. This indicated that the cells could not be defined as viable although metabolic activity was found. Based on our experimental results, *Salmonella* enters an ABNC-state after long desiccation periods under environmental conditions found in the feed industry. However, further studies are still needed to investigate whether specific environmental conditions could revive ABNC *Salmonella* cells back into a culturable state. Such studies should involve but not be limited to *in vitro* screening studies in which isolated resuscitation factors from indigenous flora, commonly found on feed processing surfaces, are tested for their involvement into reviving VBNC *Salmonella*.

This study shows that long term exposure to feed processing environmental conditions induces *Salmonella* into a non-culturable state even though about 1 % of the population remains metabolically active. It is therefore important to distinguish between the VBNC-state in which cells may revive after sub-lethal injury, and cells that in spite of showing signs of metabolic activity remain unculturable. This has significant implications when monitoring *Salmonella* from the environment which would yield false negative results using conventional pre-enrichment detection methods. These results hint on the complex unidentified conditions needed for reviving non-culturable *Salmonella* following long periods of desiccation. The need for mapping key factors involved in the persistence of *Salmonella*, would be beneficial in two major ways: *i*) by improving selective growth medium targeting non culturable cells, and *ii*) by enabling a proper risk assessment of revived non culturable cells following desiccation periods. Such feat would help improve feed safety measures in the long run.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

Two *Salmonella* strains were used in this study: a representative Norwegian feed industry isolate of *Salm.* Agona ([Nesse *et al.* 2003](#_ENREF_18)) and a laboratory strain, *Salm.* TyphimuriumATCC 14028. Both were cultivated at 30 °C in Tryptone Soy Broth (TSB; Oxoid Ltd., Hampshire, England) and Tryptone Soy Agar (TSA; Oxoid Ltd.).

**Induction of non-culturable cells via desiccation**

The experimental setup used for studying the non-culturable state of *Salmonella* was based on a previously described desiccation experimental setup ([Møretrø *et al.* 2010](#_ENREF_17)) with some modifications (cf. Supporting Information). For monitoring the induction of non-culturable *Salm.* Agona cells on coupons, sampling was performed after 1, 3, 9, 18 and 21 days incubation at dry and humid environmental conditions. Additional sampling after 30 days incubation periods at 35% and 85% RH were also performed for *Salm.* Agona and *Salm.* Typhimurium. Sampling consisted of placing individual coupons with desiccated cells in a glass tube of 5 ml saline water (150 mM NaCl). Desiccated cells were re-suspended by vortexing until the dried droplets of dessicated cells on coupons were no longer visible. The re-suspended dried cells were then used for determining the activity of non-culturable *Salmonella* through the use fluorescent probes, carboxyfluorescein diacetate (cFDA) for assessing esterase activity as well as cell membrane functionality, and the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), for quantifying respiratory activities (cf. Supporting Information). All desiccation experiments were conducted in triplicates. Direct cell counts were performed using Bürker Turk (VWR, Oslo, Norway) counting chamber and epifluorescence microscopy (cf. Supporting Information).

**Resuscitation assays of non-culturable *Salmonella* cells**

Attempts for reviving non-culturable *Salmonella* cells back into a culturable state were performed using different previously described methods. In all methods used, desiccated non-culturable cells were re-suspended in sterile peptone water prior to resuscitation assays. Revival through temperature upshifts was attempted as described ([Gupte *et al.* 2003](#_ENREF_10)), in which non-culturable cells were subjected to 80 °C for 15 s using a water bath. The method involving the use of heat stable enterobacterial autoinducers, or by plating re-suspended non-culturable cells onto sterile TSA plates, which were incubated anaerobically at 30 °C as described by Reissbrodt et al (2002) ([Reissbrodt *et al.* 2002](#_ENREF_26)), was also employed in this study.

The ability of *Salmonella* autoinducer-2 to revive non-culturable *Salmonella* cells was also investigated by re-suspending non-culturable *Salmonella* cells in pre-prepared cell-free supernatant, obtained following *Salmonella* overnight growth ([Jesudhasan *et al.* 2010](#_ENREF_14)). This was followed by incubation at 30 °C for 48 h in microwell plates placed in a Bioscreen C (Labsystems, Helsinki, Finland) reader. Optical density measurements were automatically performed every 30 min at 600 nm wavelength. Control wells consisted of either only re-suspended non-culturable cells in peptone water or wells with only cell-free supernatant.

***In-situ* scanning electron microscopy of *Salm.* Agonadesiccated cells on steel coupons**

For scanning electron microscopy (SEM), steel coupons featuring two-month old *Salm.* Agona desiccated cells incubated at 35 % RH or 85 % RH, as well as 1 h desiccated cells at room temperaturewere chemically fixated and dehydrated in 6-well plates wells (NUNC) (cf. Supporting Information).

***In vivo* infection experiments**

The preparation and harvesting of non-culturable cells used for virulence testing are described in the Supporting Information document. The *in vivo* experiment in mice was approved by the Norwegian Animal Research Authority and performed according to their guidelines. In all, 30 female inbred BALB/c mice (Charles River Laboratories, Research Models and Services, Germany) aged 8-10 weeks were included. The mice were maintained in a 12 h light/dark cycle under temperature-controlled conditions and received water and food *ad libitum*. After an acclimation period of ten days, groups of mice were infected by oral gavage (100 µl) with non-culturable *Salm.* Typhimurium(group A), culturable *Salm.* Typhimurium(groups B-E) or 0.9% NaCl (controls). Infection doses and group sizes are shown in Table 1. Following infection, the groups were kept in separate cages. They were monitored for their general health two to three times a day for up to 14 days and sacrificed by cervical dislocation if they met any early removal criteria (lethargy, hunched posture, and ruffled coat). Mice which did not display any clinical symptoms were sacrificed at the end of the experiment, except for three mice from the groups D and E which were sacrificed at day seven to see if a temporary, sub-clinical infection might be present in animals given low doses of culturable bacteria. Detailed description of the *in vivo* infection experiments can be found in the Supporting information document.

**Statistical Analysis**

Analysis of variance (ANOVA) was used to determine the variation of the number of counted injured/dead, or metabolically active cells on steel surfaces desiccated at different relative humidity environments. When needed, a one-way analysis of variance was performed to test the significance of the differences in relative humidity conditions, incubation time on desiccated *Salmonella* cells. All analyses were performed using Tukey’s test for pair wise comparisons with Minitab v15.1 (Minitab Inc., State college, PA, USA). All tests were performed at a 5 % significance level.

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**CONFLICT OF INTEREST:** no conflict of interest declared

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**Table 1:** Results from *in vivo* experiments in mice. Animals with salmonellosis all displayed clinical symptoms and *Salmonella* was isolated from the gastrointestinal tract, liver, spleen and blood.

|  |  |  |
| --- | --- | --- |
| **Mouse group** | **Infection dose** | **Nb. of mice with salmonellosis / total Nb. of mice** |
| cfu | **NC** |
| **A** | n.d. | 108 | 0/3 |
| **B** | 108 |  | 3/3 |
| **C** | 106 |  | 3/6 |
| **D** | 104 |  | 1/6 |
| **E** | 102 |  | 0/6 |
| **Control** | # |  | 0/6 |

NC = non-culturable

n.d. = not detectable, i.e. 1 cfu limit of detection

# controls were given 0.9% saline

**LEGEND TO FIGURES**

**Figure 1: Monitoring the culturable fate of desiccated *Salmonella* Agona at different environmental conditions over time.** The number of *Salm. Agona* culturable cells on TSA plates expressed as the number of *Salm.* Agona cells/ coupons was investigated over a desiccation period of 21 days at 35 % RH (◊) and 85 % RH (□) environmental conditions. Bars represent standard error of mean of three independent replicates.

**Figure 2: The effects of dry and semi-dry environmental conditions on the survival and metabolic status of *Salmonella* Agona and *Salmonella* Typhimurium cells after 30 days desiccation.** The number of*Salm.* Agona (white bars) and *Salm.* Typhimurium cells (grey bars) per coupon are presented following 30 days desiccation at 35 % RH (A) and 85 % RH (B). The number of total cells, together with cells showing signs of metabolic activity, membranes functionality, and respiratory activity were determined following enumeration using counting chamber. The number of culturable cells was determined following TSA plate count readings. Detection limit for culturable *Salmonella* was 1 cfu. Mean start counts before desiccation experiments was 8.6 and 8.4 log10 cfu/coupon for *Salm.*  Agona and *Salm.*  Typhimurium, respectively. Bars represent standard error of mean of three independent replicates.

**Figure 3: The morphological fate *Salmonella* Agona after long exposure periods to different environmental conditions.** Scanning Electron Microscopy micrographs of *Salm* Agona after 1 h desiccation on steel coupons (A), and after 2 months exposure at 35 % RH (B) and 85 % RH (C). Scale bars represent 1 µm.