**Salmonella** grows massively and aerobically in chicken faecal matter

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**Summary**

The use of wastewater for irrigation and animal manure as fertilizer can cause transmission of intestinal pathogens, conditions frequently observed in low- and middle-income countries (LMICs). Here, we tested the ability of *Salmonella* to grow in the faecal matter. We inoculated freshly isolated *Salmonella* strains (from chickens) in chicken faecal matter and incubated for 1 to 12 days, under aerobic and anaerobic conditions. We found that both *Salmonella* and *Escherichia coli* multiplied massively in faecal matter outside a host and significantly higher in aerobic conditions. Our results have critical implications in waste management, as we demonstrate that aerobic treatments may not be the best to reduce the number of *Salmonella* in the environment.

**Introduction**

Environmental transmission of intestinal pathogens is extremely important especially in low- and middle-income countries (LMICs) due to deficient sanitary infrastructure, unplanned urban growth, lack of wastewater treatment, etc. One of the main concerns in LMICs is the large proportion of untreated wastewater used for irrigation (Khalid et al., 2018) and the increasing use of animal manure as fertilizer without suitable treatment (Mandrell, 2009). Reports of grave enteric infections caused by environmental contamination of edible vegetables are also commonplace nowadays in industrialized countries (Callejón et al., 2015). Some of these outbreaks have been associated with high mortality, morbidity and large economic losses. The incidence of these infections is exacerbated by the increasing appeal to consume natural, non-processed fresh products (Mandrell, 2009).

*Salmonella*-contaminated water is responsible for a large number of outbreaks by the ingestion of water or produce (Mandrell, 2009); the sources for this contamination are human and non-human faecal matter (Medrano-Félix et al., 2017). The use of animal waste as fertilizer constitutes a serious risk that can be controlled by appropriate composting technology (Tiquia et al., 1998; Szogi et al., 2015). Human waste contamination, however, is much more difficult to monitor or control in LMICs where wastewater treatment or toilets are not available (Khalid et al., 2018). The fate of *Salmonella* in these conditions is not understood completely, although some researchers indicate that *Salmonella* enters into a viable non-culturable state outside the host (Winfield and Groisman, 2003). The reduction of the risk of this type of transmission requires an understanding of every aspect of *Salmonella* physiology in the environment outside the host (Mandrell, 2009). It is worth mentioning that *Salmonella*’s ability to grow in the faecal matter has been ignored.

It is known that *Salmonella* and other *Enterobacteriaceae* survive in faecal matter for some time and it has been shown that *Escherichia coli* (another member of the *Enterobacteriaceae*) also grows massively in faecal matter (Russell and Jarvis, 2001; Vasco et al., 2015; Sharma et al., 2019). Here, we tested *Salmonella*’s ability to grow in faecal matter in aerobic and anaerobic conditions and discuss the potential implications for faecal waste management.

**Results and discussion**

Two trials were performed with *Salmonella* Infantis inoculated in chicken faecal matter. In the first trial, we determined the growth of *Salmonella* by plate counting and by molecular detection after 0, 24, 48 and 72 h of incubation; in the second trial, we performed *Salmonella* plate counting daily, from day 0 to day 12 of incubation (Fig. 1).
In the first trial, *Salmonella* Infantis inoculated in chicken faecal matter multiplied in both aerobic and anaerobic conditions; however, the aerobic growth was significantly higher than the anaerobic growth at 48 h ($P = 1.28 \times 10^{-4}$) and 72 hrs ($P = 2.94 \times 10^{-5}$). Similarly, endogenous *E. coli* growth reached its peak after 48 h, predominantly in aerobiosis ($P = 1.92 \times 10^{-2}$) and from then on, its growth rate decreased (Fig. 2, Figs S1–S4). The growth curve of total endogenous coliforms was similar to that of *E. coli*, with a peak in aerobiosis at 48 h ($P = 1.30 \times 10^{-2}$), but their counts were higher (Fig. S5).

*Escherichia coli* had the highest specific growth rate ($\mu$) during the second day in aerobiosis ($P = 8.14 \times 10^{-5}$), decreasing in the following 24 h; *Salmonella* started fast growth at 24 h and presented significantly higher values of $\mu$ in aerobiosis than in anaerobiosis at all time intervals (for $\Delta t_1$, $\Delta t_2$ and $\Delta t_3$, $P = 7.49 \times 10^{-5}$, $6.93 \times 10^{-7}$ and $9.73 \times 10^{-3}$, respectively). Likewise, endogenous coliforms presented higher $\mu$ values in aerobiosis than in anaerobiosis after 48 h ($P = 1.83 \times 10^{-5}$) (Fig. 3).

To determine whether the above growth pattern could be applied to other *Salmonella* serovars, in the first trial we run isothermal amplification 3M™ Molecular Detection Assay 2 – *Salmonella* (MDA2SAL) at different incubation times (under aerobiosis and anaerobiosis) with 5 *Salmonella* strains (belonging to different serovars) inoculated in chicken faecal matter. The molecular assay was performed daily until day 3 after incubation (0 to 72 h). For serovars Infantis, Heidelberg, Brandenburg and Stanley, the growth peak in aerobiosis was observed at 72 h ($P = 1.19 \times 10^{-3}$), while serovar Dublin growth peak occurred at 48 h (Fig. 4, Fig. S6).

In a subsequent experiment (trial 2), no colonies of *Salmonella* in XLD or XLD with NIT were observed in aerobiosis between days 2 and 6 of incubation, probably because of a massive growth of lactose-fermenting bacteria (yellow colonies) corresponding to the commensal *Enterobacteriaceae*. Increasing *Salmonella* counts were

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**Fig. 1. Scheme of experimental procedures.** The experiments performed in trial 1 and in trial 2 are indicated. In the first trial, we determined the growth of *Salmonella* by plate counting in XLD and XLD with nitrofurantoin (NIT), and by molecular detection after 0, 24, 48 and 72 h of incubation; in the second trial, we performed *Salmonella* plate counting daily, from day 0 to day 12 of incubation.

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detected on day 7 and reached a peak on day 9 \((1.8 \times 10^8\) cells per g of faecal matter\) (Fig. S7), which coincided with a reduction in the number of lactose-fermenting bacteria colonies. On days 10 to 12, \(Salmonella\) growth was not detected, but lactose fermenters kept on growing, and gluconidase reaction indicated

\[ \text{CFUs (x 10^6) per g of fecal matter} \]

\[ \text{Time (h)} \]
that 94% of them were *E. coli*. We suspect that the massive growth of lactose-fermenting bacteria was due to a different diet used in chickens during the second trial (Shang et al., 2018). In anaerobiosis, we observed no growth of *Salmonella* or lactose-fermenting bacteria from days 1 to 12.

Growth rates of *Salmonella* and *E. coli*, at different incubation time intervals, suggested a negative correlation which may indicate antagonism between these two bacterial genera (Fig. 3). We posit that *E. coli*’s initial massive replication may limit the availability of oxygen for *Salmonella* growth; once *E. coli* growth begins to decrease, *Salmonella* grows faster. Competition between these two bacterial genera has been described previously in the gut (Barrow et al., 2015; Velazquez et al., 2019) and in ready-to-eat and fresh foods, to such an extent that some authors consider that *E. coli* may not be a good indicator of *Salmonella* (Gómez-Aldapa et al., 2013).

To investigate whether there was antagonism between *Salmonella* and *E. coli* in faeces, we inoculated equal concentrations (10⁶ cells) of *S. Infantis* and an *E. coli* (isolated from chicken) in 10 g of sterile chicken faecal matter; inoculated samples were incubated in aerobiosis and anaerobiosis for 6 days. We observed that aerobic *E. coli* growth from day 3th to 6th was significantly higher than *Salmonella*’s (P values days 3 to 6 were: 1.20 × 10⁻⁵, 1.86 × 10⁻², 1.54 × 10⁻⁶ and 5.09 × 10⁻⁵, respectively) (Fig. S8), which suggests some level of competition between these two bacteria. This finding is in agreement with previous reports (Shang et al., 2018).

There were two differences between the results of the experiments in fresh faecal matter and sterilized faecal matter: (i) the interference of *E. coli* growth occurred later in sterile faecal matter (Fig. 2, Fig. S8); and (ii) there was no difference between growth under aerobic or anaerobic conditions, except for *Salmonella* on day 5 (Fig. S9). These differences may be due to physical and chemical modifications of the faecal matter by heat sterilization; autoclaved faecal matter was drier and harder probably due to dehydration and starch gelatinization (Weurding et al., 2001). Additionally, lower water activity may protect *Salmonella* (Santos et al., 2005).

To ascertain whether the aerobic or anaerobic environments are determining factors in the growth of *Salmonella* and *E. coli* in chicken faecal matter, we inoculated fresh faecal matter with *Lactobacillus reuteri* strain *Lrr* (López et al., 2019), an anaerobic bacterium (Kandler et al., 1980; Ianniello et al., 2015), and our results showed that the growth of *Lrr* was significantly higher in anaerobiosis on days 2 and 3 (P = 4.48 × 10⁻³ and 6.86 × 10⁻⁵, respectively) (Fig. S10), which is an additional evidence that the presence or absence of oxygen in the environment is a factor that determines the differential growth of *Salmonella* and *E. coli* in fresh chicken faeces. On day 6, we observed that *Lrr* growth in aerobiosis and anaerobiosis produced the same numbers of colonies; we speculate that aerotolerant mutant bacteria may have been selected during the incubation period, a phenomenon described previously in *Lactobacillus* (Ianniello et al., 2015).
Our results indicate that *Salmonella* and other *Enterobacteriaceae* multiply massively and aerobically in fresh chicken faecal matter; in fact, faecal matter incubated under aerobic conditions has more *Salmonella* (on average 10 times more) than freshly released faeces. Our results show clear evidence that the faecal matter is a transient but very important component of the *Enterobacteriaceae* life cycle, where enterobacterial population expands (Russell and Jarvis, 2001; Vasco et al., 2015; Barrera et al., 2018) increasing the chances of reaching other hosts.

Previous studies have shown that *E. coli* has a negative growth rate outside the host, with a short half-life (1 day in water, 1.5 days in sediment and 3 days in soil) (Winfield and Groisman, 2003); however, we have found that as long as it remains in faecal matter, *E. coli* continues to grow up to 12 days after being excreted in the environment (intermediate habitat) (Barrera et al., 2018). Also, it has been estimated that the doubling time of *E. coli* in its primary habitat (the intestine of warm-blooded animals) is 2 days (Winfield and Groisman, 2003), and our results indicate that its doubling time in the intermediate habitat during the first two days is less than 24 h (Fig. 2, Fig. S1). Our findings disagree with the notion that these bacteria enter a viable but not culturable status when excreted from the host (Winfield and Groisman, 2003). Additional studies are needed due to the relevance of this issue in public health.

Microbiologists have struggled to explain why bacteria adapted to the anaerobic intestinal milieu possess energetically costly machinery to use oxygen (Govantes et al., 2000). Further, it has been shown that aerobic respiration is not important for *Salmonella* intestinal colonisation (Barrow et al., 2015). We hypothesize that the reason for this apparent evolutionary mystery may be related to the enterobacterial ability to grow in faecal matter under aerobic conditions. *Enterobacteriaceae* are facultative anaerobe which can synthesize ATP by different enzymatic pathways, depending on the external concentration of O$_2$ and the redox changes in the environment. When O$_2$ is available, the bacteria obtain energy by aerobic respiration, with O$_2$ being the final acceptor of electrons. In shortage of O$_2$, these bacteria generate ATP by one of the following mechanisms: (i) synthesis of terminal oxidases that allow the bacteria to take advantage of traces of O$_2$; (ii) use of other inorganic molecules (such as NO$_3^-$ and S$_2$O$_8^{2-}$) as final electron acceptors (Yamamoto and Droffner, 1985; Bueno et al., 2012; Rivera et al., 2013); and (iii) use of organic compounds as donors and acceptors (Madigan et al., 2012). However, aerobic respiration produces much better performance in terms of ATP molecules per substrate molecule (Madigan et al., 2012).

*Salmonella* is responsible for hospitalizations and deaths worldwide (Omer et al., 2018; EFSA and ECDC, 2019) due to outbreaks associated not only with animal products but also with vegetables (Gunel et al., 2015; Omer et al., 2018). The presence of *Salmonella* in produce is associated with unintended environmental faecal contamination and the use of untreated manure as fertilizer (Fletcher et al., 2013). Our results have critical implications in waste management, contribute to select more efficient ways of treating manure through composting (Singh et al., 2012; Román et al., 2015) and suggest the need of anaerobic treatments for animal waste.

The loose consistency of avian faeces allows the entry of air, and this phenomenon may contribute to the proficiency of these animals to spread *Salmonella*. Similarly, loose stools caused by *Salmonella* infection may favour the growth of this bacterium in faecal matter from animals with different faecal texture.

The inconsistencies found in this study are probably due to the complex composition of faecal matter (food substrates and microbiota). Another limitation was the abundant growth of accompanying bacteria (lactose fermenters) that made difficult the detection of *Salmonella* in XLD.

This type of studies is important because it helps to understand better the physiology of *Salmonella* and other members of the *Enterobacteriaceae* family. We addressed a neglected but crucial characteristic of *Salmonella* life cycle which may have an impact in public health.

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**Conflict of interest**

The authors declare no conflict of interest.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Data points of the growth of Salmonella Infantis inoculated in chicken fecal matter, under aerobic conditions. Typical Salmonella colonies were counted in XLD and XLD with NIT (12 mg l\(^{-1}\)). The number of Petri dishes counted is represented by \(n\); for 0 and 72 h \(n = 14\), for 24 h \(n = 15\), and for 48 h \(n = 6\). The experiment was performed twice and correspond to the first trial.

Fig. S2. Data points of the growth of Salmonella Infantis inoculated in chicken fecal matter, under anaerobic conditions. Typical Salmonella colonies were counted in XLD and XLD with NIT (12 mg l\(^{-1}\)). The number of Petri dishes counted is represented by \(n\); for 0 and 48 h \(n = 14\), for 24 h \(n = 16\), and for 72 h \(n = 10\). The experiment was performed twice and correspond to the first trial.

Fig. S3. Data points of the growth of endogenous E. coli in chicken fecal matter, under aerobic conditions. E. coli was counted in 3M™ Petrifilm E. coli/Coliform Count Plates. The number of Petri dishes counted is represented by \(n\); for 0 h \(n = 6\), for 24 h \(n = 7\), for 48 and 72 h \(n = 4\). The experiment was performed twice and correspond to the first trial.

Fig. S4. Data points of the growth of endogenous E. coli in chicken fecal matter, under anaerobic conditions. E. coli was counted in 3M™ Petrifilm E. coli/Coliform Count Plates. The number of Petri dishes counted is represented by \(n\); for 0, 24 and 72 h \(n = 6\), for 48 h \(n = 8\). The experiment was performed twice and correspond to the first trial.

Fig. S5. Growth of endogenous total coliforms in chicken fecal matter, under aerobic and anaerobic conditions. The number of total coliforms corresponded to the sum of the red and blue colonies with gas in 3M™ Petrifilm E. coli/Coliform Count Plates incubated 24 and 48 h. Data shown are means ± SD. Asterisk indicates statistically significant difference (t-test, \(P < 0.05\)) between aerobic and anaerobic growth. The number of Petri dishes counted is represented by \(n\); for 0 h \(n = 8\); for 24 h aerobicosis and anaerobiosis, 48 h aerobicosis and 72 h anaerobiosis \(n = 4\); for 72 h aerobicosis \(n = 6\); and for 48 h anaerobiosis \(n = 3\). The experiment was performed twice and correspond to the first trial.

Fig. S6. Individual growth curves of Salmonella serovars. These curves were obtained by 3M™ Molecular Detection Assay 2 - Salmonella (MDA2SAL). The blue lines correspond to the growth under aerobic conditions and red ones, under anaerobic conditions. The number of independent readings is represented by \(n\); for all data points \(n = 1\). The experiment was performed once and correspond to the first trial.

Fig. S7. Data points of the growth of Salmonella Infantis inoculated in chicken fecal matter, under aerobic conditions, days 0 to 12. Typical Salmonella colonies were counted in XLD and XLD with NIT (12 mg l\(^{-1}\)). This graph considers the results of the first trial (2 repetitions) and the second trial (1 repetition). The number of Petri dishes counted is represented by \(n\). For 0 days \(n = 17\), for 1 day \(n = 16\), for 2 and 9 days \(n = 6\), for 3 days \(n = 14\), for 7 days \(n = 1\) and for 8 days \(n = 2\).

Fig. S8. Growth curves of Salmonella Infantis and E. coli inoculated in sterile fecal matter, under aerobic conditions. Colonies were counted in MKL. Data shown are means ± SD. Asterisks indicate statistically significant difference (t-test, \(P < 0.05\)) between the number of Salmonella and E. coli. The number of Petri dishes counted is represented by \(n\). For Salmonella \(n = 4\), except on days 1 (\(n = 3\)) and 2 (\(n = 2\)). For E. coli \(n = 4\), except on day 1 (\(n = 3\)). The experiment was performed once.

Fig. S9. Growth curves of Salmonella Infantis and E. coli inoculated in sterile fecal matter, under aerobic and anaerobic conditions. Colonies were counted in MKL. Data shown are means ± SD. Asterisk indicates statistically significant difference (t-test, \(P < 0.05\)) between aerobic and anaerobic growth. The number of Petri dishes counted is represented by \(n\). For Salmonella \(n = 4\), except on day 1 aerobicosis and anaerobiosis, and day 3 anaerobiosis (\(n = 3\)), day 2 aerobicosis and anaerobiosis (\(n = 2\)) and day 6 anaerobiosis (\(n = 8\)). For E. coli \(n = 4\), except on day 1 aerobicosis and anaerobiosis (\(n = 3\)), day 2 aerobicosis (\(n = 2\)) and day 6 anaerobiosis (\(n = 8\)). The experiment was performed once.

Fig. S10. Growth curves of Lactobacillus reuteri rifampicin resistant in chicken fecal matter, under aerobic and anaerobic conditions. Colonies were counted in MRS agar + Rifampicin (100 \(\mu g\) ml\(^{-1}\)). The brown line corresponds to the growth under aerobicosis and the blue one, under anaerobiosis. Data shown are means ± SD. Asterisks indicate statistically significant difference (t-test, \(P < 0.05\)) between aerobic and anaerobic growth. The number of Petri dishes counted is represented by \(n\). For the data points \(n = 4\), except for day 3 aerobicosis (\(n = 3\)) and day 6 (\(n = 6\)). The experiment was performed once.

Data S1: Experimental Procedures.