**Title:** Comparative phenotypic, genomic and transcriptomic characterisation of two *Salmonella* Typhimurium strains for a first-in-human challenge model

**Short Title**: *Salmonella* Typhimurium CHIM strain characterisation

**Authors**

Christopher Smith[1 ¶ *], Anan Bzami[2¶], Xiaojun Zhu[3¶], Jessica A. White[2], Nerie Roa[2], Akamol E. Suvarnapunya[4], Emma Smith[1], Anna Rydlova[1], Robert Varro[1], Zainab Kiliddar[5], Lijuan Luo[3], Blanca Perez Sepulveda[3], Melita A. Gordon[3], Graham S. Cooke[1], Robert K.M. Choy[3], Jay C.D. Hinton[3], Malick M. Gibani[1]

**Affiliations**

[1] Department of Infectious Disease, Imperial College London, London, UK

[2] PATH, Seattle, WA, USA

[3] Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, UK

[4] Department of Diarrheal Disease Research, Walter Reed Army Institute of Research, Silver Spring, MD, USA

[5] North-West London Pathology, Imperial College Healthcare NHS Trust, London, UK

[¶] Authors contributed equally

[*]**Corresponding Author:** Department of Infectious Disease, Sir Alexander Fleming Building, South Kensington Campus, Imperial College London, London SW7 2AZ

**Keywords:** *Salmonella*; *Salmonella* Typhimurium; Nontyphoidal *Salmonella*; Human Challenge Model; Controlled Human Infection Model; Vaccines.

## ABSTRACT

Invasive nontyphoidal *Salmonella* (iNTS) disease remains a major public health challenge in sub-Saharan Africa. *Salmonella* Typhimurium (STm) is responsible for the majority of cases, with specific lineages being associated with increased risk of bloodstream infection. We have recently developed a STm controlled human infection model (CHIM) to better understand disease pathogenesis and to provide a platform to test candidate vaccines. Selecting appropriate challenge strains is a central design consideration in developing challenge model protocols. We describe the rationale, manufacture, and detailed characterisation of two STm strains used in the first-in-human NTS CHIM.

Two well-characterised STm strains were selected: 4/74 (ST19 lineage), a globally distributed diarrhoeal isolate, and D23580 (ST313 lineage 2.0), the predominant invasive pathovariant in Africa. These strains were selected based on phylogenetic relevance, antibiotic susceptibility, known provenance, and comprehensive existing virulence data. Manufacturing was conducted under Good Manufacturing Practice (GMP) conditions, with confirmation of purity, viability, and stability. Phenotypic characterisation included growth profiling, motility, colony morphology, acid and buffer sensitivity, and antibiotic susceptibility. Whole-genome sequencing confirmed genetic stability. Additionally, we investigated the effect of sodium bicarbonate exposure - used for gastric acid neutralisation - on STm virulence gene expression using transcriptomic analysis.

Both strains retained expected *in vitro* phenotypic characteristics, including reduced motility and melibiose utilisation in D23580. Growth and viability were preserved in simulated gastrointestinal conditions and holding buffers. No unexpected mutations were detected, and both strains remained susceptible to study antibiotics. Transcriptomic profiling revealed no

51 significant changes in key *Salmonella* pathogenicity island genes after sodium bicarbonate
52 exposure, suggesting unaltered virulence at administration.
53
54 These findings confirm that STm 4/74 and D23580 retain genomic and phenotypic integrity post-
55 manufacture. The resulting challenge stocks provide the foundation for an ongoing NTS CHIM
56 that aims to advance understanding of NTS pathogenesis and support candidate vaccine testing.

## AUTHOR SUMMARY

Nontyphoidal *Salmonella* (NTS) infections cause significant illness worldwide, particularly in sub-Saharan Africa, where invasive NTS (iNTS) disease leads to life-threatening bloodstream infections. Developing vaccines to prevent iNTS is a public health priority, but progress has been slow due to gaps in understanding how these bacteria cause disease and how the immune system responds. Controlled human infection models (CHIMs) can accelerate vaccine development by allowing detailed investigation of infection biology in a controlled setting.

This study describes the selection, production, and detailed characterisation of two *Salmonella* Typhimurium strains for the first-in-human NTS CHIM. The strains – one associated with diarrhoeal disease and the other with increased risk of bloodstream infection – were chosen based on their global relevance and comprehensive prior testing. We confirmed their stability, antibiotic susceptibility, and behaviour in conditions mimicking the human gastrointestinal tract. Importantly, we found that sodium bicarbonate, used to neutralise stomach acid before infection, does not alter bacterial virulence.

These findings support and build on existing characterisation data for two *S*. Typhimurium strains which account for a significant proportion of NTS infections globally. This CHIM will be a valuable platform for improving our understanding of NTS infection biology and testing new iNTS candidate vaccines.

# 1 INTRODUCTION

Invasive nontyphoidal *Salmonella* (iNTS) is a major cause of community-acquired bloodstream infection in sub-Saharan Africa (sSA) and is associated with significant morbidity and mortality [1]. iNTS disease was estimated to have caused 535,000 cases and 77,500 deaths in 2017 [2]. The greatest burden is observed in children under 5 years, with recognised risk factors including anaemia, malnutrition, and recent malaria infection [3]. Immunocompromised adults with HIV are also susceptible; however, incidence of HIV-associated infection has been declining with improved access to effective antiretroviral therapy [4].

*Salmonella* Typhimurium (STm) is the most commonly isolated serovar in iNTS bloodstream infections, followed by *S*. Enteritidis (SEn) [5]. *S*. Typhimurium multi-locus sequence type (ST)313 has been identified as the dominant circulating pathovar in sSA and is associated with multi-drug resistance to amoxicillin, chloramphenicol and co-trimoxazole [5,6]. In addition to challenges posed by antimicrobial resistance (AMR), public health interventions to control iNTS disease burden are hampered by an incomplete understanding of transmission reservoirs and a lack of preventative vaccines [7]. Several promising vaccine candidates are in early-stage human development, including OAg/FliC conjugate vaccines and those utilising the Generalised Modules for Membrane Antigens (GMMA) platform [8–10]. The leading candidate vaccines are likely to progress as multivalent formulations targeting both STm and SEn, potentially alongside antigenic targets for typhoidal *Salmonella*.

Controlled human infection models (CHIMs) can provide unique insights into mechanisms of disease pathogenesis and elucidate immune correlates of protection [11]. In some circumstances, CHIMs can also provide clinically relevant platforms for candidate vaccine efficacy studies, supporting licensure decisions [12]. This has been particularly true for enteric

104 pathogens, where CHIMs have played a central role in accelerating development of vaccines
105 against *S*. Typhi and *V. cholerae* [13,14].
106
107 We have recently established a first-in-human NTS CHIM. This model aims to characterise the
108 clinical and microbiological responses of healthy volunteers to oral challenge with *S*.
109 Typhimurium, describe putative immune correlates of protection, and provide a platform for
110 future NTS vaccine efficacy assessments. A consortium of experts in the fields of *Salmonella*
111 biology, epidemiology, vaccine development and human challenge models have contributed to
112 the study design [15].
113
114 Several considerations relating to bacterial challenge strain selection, manufacture and
115 characterisation are central to the safe and ethical conduct of controlled human infection
116 models. The approach to strain selection has been described in detail for other bacterial human
117 challenge models [16,17]. Whilst some pathogen-specific considerations may apply, some
118 general principles are outlined in **Table 1**. To maximise the utility of a disease model, challenge
119 strains should aim to be as a minimum: i) phylogenetically representative of contemporary
120 disease-causing isolates; ii) susceptible to commonly used antibiotics; iii) have a traceable
121 history from isolation; iv) possess a representative repertoire of virulence factors and v) be
122 associated with a relevant disease phenotype for vaccine efficacy assessment. Here we describe
123 the strategy used to select the most relevant strains for use in a novel NTS CHIM. We provide
124 comprehensive phenotypic and genomic characterisation of selected strains and detail the
125 testing protocols associated with manufacture to GMP standards. We go on to explore the effect
126 of sodium bicarbonate exposure on *S*. Typhimurium virulence gene expression, in an attempt to
127 model the bacterial transcriptional profile at the point of oral administration.

| Consideration | Rationale |
|---|---|
| Challenge Agent Strain Selection | • Representative of current strains causing natural infection or disease by the pathogen under investigation<br>• Selected to produce measurable clinical symptoms or signs which mimic natural infection with the pathogen under investigation (established by pre-determined clinical endpoints), without causing severe disease or complications.<br>• Wild type, attenuated, or genetically modified strains may be utilised, determined by the primary objective of the CHIM and its intended application e.g. vaccine efficacy assessment.<br>• Full provenance of the challenge agent must be known and should have a traceable history from isolation |
| Challenge Agent Strain Characteristics | • Reliable susceptibility to antibiotics intended for use within the context of a CHIM<br>• Demonstrated genetic stability throughout the study period to avoid unexpected changes in virulence or resistance |
| Challenge Agent Strain Manufacture, QC and Testing | • Equipment and reagents must be available, serviced, calibrated, and operated by trained personnel<br>• Production should be within laboratory facilities with appropriate Biosafety containment level suitable for the specific CA<br>• Key physical and chemical properties of the CA must be demonstrated and recorded: identification via standard microbiological diagnostics, absence of impurities or toxins, stability and sensitivity to pH, temperature chemicals<br>• Purity: CA should be free from other agents of human infection (viruses, bacteria, fungi), determined by microbial limits testing<br>• Stability: CA should maintain stability following manufacture, transport and storage, determined by repeat testing and appropriate intervals |
| Regulatory Compliance | • CA dossier must be produced and available following release from manufacture, detailing testing results from the above listed requirements<br>• Engagement with regulatory bodies overseeing manufacture and production is essential and comply with local/national regulations e.g. international variation in classification of CAs as investigational medical products<br>• Full consideration must be given to public health and infection control aspects of CA use e.g. informing local health protection teams and developing risk assessments to manage unintentional release or transmission of CA<br>• Risk assessments must be in place for the import, transport and storage of CA within appropriate biosafety facilities |

**Table 1:** Key considerations for challenge agent selection, development and production for controlled human infection models. CA: challenge agent, CHIM: controlled human infection model, QC: quality control. Adapted from [18].

# 2 RESULTS

## 2.1 Challenge Agent Strain Selection

Strain selection for use in the CHANTS study has been conducted in accordance with the principles and considerations outlined in **Table 1**. iNTS in sSA is known to occur in at-risk populations with specific risk factors, such as recent malaria, malnutrition, sickle cell disease and advanced HIV infection. Additionally, the dominant circulating *S*. Typhimurium strains responsible for the majority of infections have been shown to have undergone evolutionary changes, characterised by genomic degradation and pseudogene formation, which are postulated to represent host adaptation towards an invasive phenotype [6]. The relative contributions of host susceptibility and host-adapted bacterial factors in iNTS are incompletely understood.

To address some of these knowledge gaps, the CHANTS study utilises two distinct strains of *S*. Typhimurium which are considered representative of both invasive (iNTS) and diarrhoeal (dNTS) disease-causing isolates. ST313 is the dominant iNTS pathovar in sSA, whilst ST19 is considered an archetypal strain representative of globally distributed dNTS infections [5]. In addition to their epidemiological relevance, D23580 (ST313) and 4/74 (ST19) are model organisms which have a traceable history from isolation, and have undergone extensive prior *in vitro* phenotypic, genomic, and transcriptomic characterisation, which facilitates a deeper understanding of biological properties [19]. Both strains have been deposited in the UK Health Security Agency (UKHSA) National Collection of Type Cultures (NCTC) and have been sourced from this repository for the purposes of this study (D2350 [Ref 14677]; 4/74 [Ref 14672]).

156 The final selection of these specific strains was informed at an expert consultation meeting, and
157 was ultimately based on their status as well-characterised archetypal laboratory strains
158 representing two major lineages of *Salmonella* Typhimurium [15].
159

160 **2.2 GMP manufacture, quantification, and purity of challenge stocks**
161

162 Identification of manufactured challenge agent stocks was initially confirmed by positive
163 agglutination of both strains with *Salmonella* group B antiserum. Pre- and post-freeze viability
164 was confirmed by colony counting. The pre-freeze viability of ST19 4/74 was 1.24 x $10^9$ CFU/ml
165 and the post-freeze viability was 5.2 x $10^8$ CFU/ml. The pre-freeze viability of ST313 D23580 was
166 9.95 x $10^8$ CFU/ml, and the post-freeze viability was 4.9 x $10^8$ CFU/ml.
167

168 Microbial limits testing confirmed manufactured challenge agent stocks were free from non-
169 sterile products, including specified organisms: Bile Tolerant Gram-Negative bacteria,
170 *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus spp., Clostridia*
171 *spp., Aspergillus spp.*, and *Candida spp*. Stocks diluted in TSB diluent $10^{-3}$ to $10^{-8}$ and plated on a
172 range of organism specific selective media confirmed absence of objectionable organisms.
173

174 **2.3 *S.* Typhimurium viability is reduced in acidic pH <3.5**
175

176 We next evaluated the optimal conditions for challenge agent viability and delivery, focusing on
177 the role of gastric acid pH as a first line of defence against enteric pathogens. Previous *S.* Typhi
178 challenge studies demonstrated that neutralizing gastric acid prior to administration significantly
179 lowers the infectious $ID_{50}$ dose [20]. Pre-treatment with sodium bicarbonate – routinely used in
180 human challenge models for *S.* Typhi [20], *Vibrio cholerae* [21], and *Shigella* spp. [22] is also
181 thought to reduce batch-to-batch variability and enhance reproducibility in enteric challenge
182 studies.

183 We began by assessing the pH sensitivity of strains D23580 and 4/74, with the aim of defining a
184 threshold below which viability of the challenge strains significantly decreases. $5.15 \times 10^5$ CFU/ml
185 of each strain were incubated for 15 minutes in several pH test conditions prior to plating. Both
186 strains demonstrated similar stability in 0.9% saline, sodium bicarbonate solution (2.6g/120ml),
187 and acidic conditions above pH 4. As expected, survival significantly declined following
188 incubation in pH ≤ 3.5, with D23580 demonstrating greater sensitivity in this pH range (**Figure 1**).
189 These findings are consistent with known sensitivity of *Salmonella* to acidic conditions and
190 supports the commonly utilised approach of neutralising gastric acid with bicarbonate pre-
191 treatment in enteric challenge models [23,24].
192

193 **2.4 *S.* Typhimurium viability remains stable in 0.9% saline and bicarbonate buffer to 1**
194 **hour**
195

196 We sought to determine the stability of challenge strains under various holding buffer conditions.
197 We began by comparing bacterial survival following incubation in 0.9% saline and sodium
198 bicarbonate (2.6g/120ml) buffer, in addition to testing different holding times on ice (T = 0, 1, 2, 3
199 hours) to determine the optimal solution and maximum hold duration to ensure viability at the
200 time of oral administration. Survival was maintained at >90% in 0.9% saline solution to T=2hrs
201 and dropped significantly to <80% after T=3hrs, compared with T=0hr baseline. Survival in
202 sodium bicarbonate was reduced in comparison, with a marked drop in survival <80% at T=2hrs
203 (**Figure 2**). These findings were initially demonstrated with D23580 and subsequently confirmed
204 with repeat testing on 4/74 (T = 1 hour; saline only). As a result of these findings, we updated the
205 challenge agent preparation SOP to use 0.9% saline instead of sodium bicarbonate as the
206 holding buffer with a maximum one-hour hold interval between formulation and administration.

207 ## 2.5 Sodium bicarbonate pre-treatment maintains simulated gastric fluid pH above
208 critical thresholds for STm survival
209

210 In the *Salmonella* Typhimurium challenge (CHANTS) study, participants are administered a
211 challenge agent in the form of a liquid suspension after fasting for at least 90 minutes. The
212 challenge procedure comprises two components: (i) a sodium bicarbonate solution (2.6g/120ml)
213 pre-treatment for gastric acid neutralisation, followed by (ii) a 30ml 0.9% saline solution-
214 bacterial challenge agent suspension.
215
216 We next evaluated the impact of sodium bicarbonate pre-treatment on the expected pH of the
217 stomach at the time of bacterial challenge. Gastric acid represents a key defence mechanism
218 against gastrointestinal infection. *Salmonella enterica* have evolved multiple strategies to
219 improve survival following acid exposure, including the acid-tolerant response (ATR) and the
220 arginine D-carboxylase system, but the organism still remains susceptible to gastric acid killing
221 [23–25].
222
223 To model the gastric passage of the liquid challenge agent, we used the Rosset-Rice (RR) *in vitro*
224 system to model bacterial stability and survival following simulated transit through the stomach
225 and intestinal tract. The RR model is designed to replicate the low pH conditions of a fasted adult
226 stomach by introducing acid at rates that mimic physiological acid secretion. The model is
227 predicated on a set of assumptions concerning gastric physiology. In particular, the rate of
228 gastric emptying is known to be regulated by several factors (25). In fasted adults, it is estimated
229 that 240ml volume of non-caloric liquid can empty from the stomach in approximately 15-20
230 minutes [26,27]. The mean residual volume in a stomach after an overnight fast is estimated to
231 range between 25-50ml [26,28,29]. Allowing for inter-individual variability, the resting pH of a
232 fasted adult stomach typically ranges from pH 1 to 3 [30]. The basal acid output in healthy adult
233 stomachs is estimated to be 1-5 mmol HCl/hour, which increases significantly on eating [31].

234 Although it mimics some key aspects of gastric physiology, one limitation is that it does not
235 simulate the removal of gastric contents which would usually dilute the acidic environment as
236 contents are removed over time.
237
238 Gastric pH dynamics were modelled in the modified Rossett-Rice *in vitro* system modelling an
239 antacid dose of 2.6g sodium bicarbonate buffer to ensure that it maintains pH above the critical
240 threshold of pH 3.5 identified for D23580 and 4/74 survival. Serial measurements every minute
241 confirmed that gastric pH is maintained above the critical threshold pH >3.5 and is approximately
242 pH 6.5 at 70 minutes following administration of bicarbonate buffer (**Figure 3**).
243

244 **2.6 *S.* Typhimurium viability remains stable in simulated gastric and intestinal fluids**
245 **after 1 hour hold on ice**
246

247 The modified Rossett-Rice model was then utilised to determine bacterial stability and survival
248 following simulated transit through the gastrointestinal tract. STm challenge strains were
249 incubated in 0.9% saline solution and either directly added to the RR model or after a 1hour hold
250 on ice. Fluid was then sampled at 0, 30, 45 and 60 minutes after addition to the RR model and
251 plated for overnight incubation. There were no significant differences in survival between T=0hr
252 and T=1hr hold on ice prior to addition to the RR model. CFU counts declined with time and
253 overall survival was approximately 70% and 80% after 60 mins in the RR model for 4/74 and
254 D23580, respectively (**Figure 4**). We further modelled survival of the challenge strains during
255 transit from the stomach to the small intestine. STm challenge strains were again subjected to
256 the RR model and after 70 minutes samples were taken and exposed to simulated intestinal fluid
257 and subsequently plated at 0, 30, 70-minute time points to mimic residence time in the upper
258 small intestine. There was no difference in survival of either challenge strain between 0-70
259 minutes of exposure to simulated intestinal fluid.
260

## 2.7 Phenotypic Confirmation and Characterisation

### 2.7.1 STm D23580 and 4/74 exhibit differential growth characteristics in melibiose

A key metabolic property distinguishing D23580 from 4/74 is the differential ability to utilise disaccharide melibiose as a sole carbon source [19]. D23580 contains three nonsynonymous SNPs in melibiose utilisation genes (*melB* and *melR*) leading to downregulated expression of proteins responsible for active transport of melibiose across the cell membrane. It is speculated that the ability of 4/74 to metabolise melibiose confers a survival advantage during gastrointestinal colonisation, and the loss of this capacity in D23580 reflects niche adaptation towards an invasive phenotype [19]. This differential utilisation was demonstrated by evaluation of bacterial growth curves in minimal media and LB. Growth curves were comparable between strains in minimal media supplemented with 5% glucose, whilst growth of STm D23580 is markedly attenuated in minimal media supplemented with melibiose (**Figure 5**).

### 2.7.2 STm D23580 and 4/74 exhibit differential swimming motility

As previously described, swimming motility in soft agar is reduced in D23580 compared with 4/74 due to downregulation of flagellin expression. This phenotype is consistent with other invasive *Salmonella* serovars including Typhi, Paratyphi A, and Gallinarum, which rapidly reduce flagellin expression upon cellular invasion to avoid immune recognition by TLR-5 and flagellin-specific $CD4^+$ T-cells [32–34]. The reduced swimming motility of D23580 compared with 4/74 in soft agar after 4 hours incubation at 37°C is illustrated in **Figure 6**.

### 2.7.3 STm D23580 and 4/74 exhibit differential RDAR morphotypes

At room temperature, many strains of *Salmonella* can form red, dry, and rough (RDAR) colonies, which result from the formation of aggregative fimbriae. RDAR colonies reflect multicellular

289 behaviour that enhances *Salmonella* environmental stress resistance and biofilm formation.
290 This loss of multicellular behaviour has been demonstrated in D23580, hypothesised to occur as
291 a consequence of human host-adaptation [35]. A positive RDAR phenotype appears as a red
292 colony with filamentous texture, whilst a negative RDAR phenotype appears as a smooth and
293 yellow colony. Differential RDAR morphotype appearances between 4/74 and D23580 are
294 illustrated in **Figure 6**.
295

296 **2.7.4 Antibiotic Susceptibility**
297

298 Multi-drug resistance in *Salmonella spp*. is defined as resistance to ampicillin, co-trimoxazole
299 and chloramphenicol. iNTS is increasingly multi-drug resistant in Africa, demonstrated by the
300 emergence and persistence of ST313 [5]. D23580 harbours four plasmids, including pSLT-BT,
301 which encodes resistance to chloramphenicol, ampicillin, streptomycin, sulphonamides, and
302 trimethoprim.
303

304 Disc diffusion on Mueller-Hinton agar, and broth microdilution using BD Phoenix provided zone
305 inhibition diameters and MIC concentrations respectively and were compared to published
306 EUCAST guidelines for sensitivity [36]. Antibiotic sensitivity results for both strains were as
307 predicted in both disc diffusion and broth microdilution methods. BD Phoenix minimum
308 inhibitory concentrations (MIC) and interpretations are illustrated in **Table 2**.
309

| Antimicrobial | MIC | Interpretation |
|---|---|---|
| **4/74 (ST19)** | | |
| Ampicillin | <=2 | **S** |
| Amoxicillin-Clavulanate | <=2/2 | **S** |
| Ceftriaxone | <=0.5 | **S** |
| Ciprofloxacin | <=0.25 | **S** |
| Gentamicin | <=1 | **S** |
| Meropenem | <=0.125 | **S** |
| Piperacillin-Tazobactam | <=4/4 | **S** |
| Trimethoprim-Sulphamethoxazole | <=1/19 | **S** |
| **D23580 (ST313)** | | |
| Ampicillin | >8 | **R** |
| Amoxicillin-Clavulanate | 16/2 | **R** |
| Ceftriaxone | <=0.5 | **S** |
| Ciprofloxacin | <=0.25 | **S** |
| Gentamicin | 2 | **S** |
| Meropenem | <=0.125 | **S** |
| Piperacillin-Tazobactam | <=4/4 | **S** |
| Trimethoprim-Sulphamethoxazole | >4/76 | **R** |

**Table 2**: Antibiotic susceptibility profiles of STm 4/74 and D23580 as measured by broth microdilution using the automated BD Phoenix system. Minimum inhibitory concentrations (MIC) were measured and interpreted in accordance with EUCAST guidelines to determine susceptibility. S = susceptible, R = resistant

314 **2.8 Whole genome sequencing confirms STm genomic stability post-manufacture**
315

316 Illumina whole-genome sequencing was performed to assess for single nucleotide
317 polymorphism (SNP) differences which may have arisen during the manufacturing process
318 compared with established reference strain sequences.
319

320 No SNP mutations were detected comparing *S*. Typhimurium 4/74 or D23580 manufactured
321 stocks with the reference strain sequences [4/74 Genbank Accession Numbers: CP002487.1 –
322 CP002490.1; D23580 Genbank Accession Numbers: LS997973.1 – LS997977.1], highlighting
323 overall genomic stability. Consistency with genetic sequences from reference strains ensures
324 the manufactured stocks administered to participants express the same repertoire of virulence
325 factors as circulating strains causing iNTS in sSA. Moreover, these strains express the putative
326 key antigens targeted by vaccine candidates in development, including the *wbaV, wbaU, wbaN,*
327 *wzxB1* genes in the O-antigen biosynthesis cluster encoding O4, alongside *fliC* and *fliB* genes
328 encoding phase variable flagellar antigens.
329

330 **2.9 Sodium bicarbonate exposure does not impact key STm virulence gene expression**
331

332 Administration of sodium bicarbonate pre-treatment is frequently utilised in enteric pathogen
333 CHIMs to reduce the required challenge inoculum, and to reduce batch-to-batch variation
334 [20,37,38]. We have demonstrated that STm challenge strain survival is stable, and gastric pH is
335 maintained above critical thresholds in a simulated gastrointestinal tract. However, sodium
336 bicarbonate exposure has also been shown to have a significant effect on bacterial virulence
337 factor gene expression in other enteric pathogens, notably in *E. coli* spp, *Citrobacter,* and *V.*
338 *cholerae* [39–42]. We sought to investigate the impact of sodium bicarbonate exposure on *S.*
339 Typhimurium virulence gene expression, which may alter the virulence of challenge agent stocks
340 at the time of oral administration.

341 Transcriptomic profiling of STm D23580 and 4/74 has previously been undertaken and
342 characterised in multiple infection-relevant conditions [19]. In this investigation, we performed
343 RNA-seq on whole RNA extracted from STm D23580 and 4/74 after 15-, and 30-minutes
344 incubation in sodium bicarbonate solution (0.53g/30ml) and compared to gene expression levels
345 after incubation in water. These conditions were selected for testing to reflect the anticipated
346 duration bacterial cells will be in contact with bicarbonate buffer during transit from the stomach
347 to the small intestine following oral challenge.
348
349 Overall transcriptome of both strains was evaluated in these 3 conditions, and individual gene
350 expression level calculated as transcripts per million (TPM). A gene is considered as expressed
351 when TPM is greater than 10. Both strains 4/74 and D23580 showed a decreasing ratio of
352 expressed genes with increasing time of incubation in sodium bicarbonate solution (4.11% for
353 4/74 and 8.15% for D23580). Both strains expressed approximately 70% genes after 30 minutes
354 incubation in sodium bicarbonate solution compared with water.
355
356 *Salmonella* pathogenicity islands SPI-1 and SPI-2 play major role in human infection [43,44],
357 where SPI-1 plays a role in bacterial invasion to epithelial cells, whilst SPI-2 genes regulate
358 formation of *Salmonella*-containing vacuoles (SCVs) [45]. Flagella are also major virulence
359 factors during *Salmonella* infection [46]. It has been shown that sodium bicarbonate exposure
360 down-regulates flagella expression in *E. coli* [42]. To determine the impact of sodium bicarbonate
361 exposure on *Salmonella* virulence gene expression, we used a differential expression approach
362 to compare gene expression before and after sodium bicarbonate treatment and determined that
363 6% of 4/74 genes and 5% of D23580 genes were differentially expressed. No significant up-
364 regulation or down-regulation was detected in SPI-1, SPI-2 or flagellar genes in either strain
365 following bicarbonate exposure. To determine the likely functional impact of these up- or down-
366 regulated genes, we examined gene ontology and determined that there was no common

367 functional links among the genes (**Figure 7**). This suggests that minor changes in gene expression
368 likely reflect experimental variability rather than a meaningful biological trend. In summary, these
369 transcriptomic data suggest that sodium bicarbonate exposure does not have a significant effect
370 upon *Salmonella* virulence.
371

372 # 3 DISCUSSION

373

374 Bacterial stocks used for controlled human infection studies need to be manufactured to a high
375 standard. In this study, we have described the processes involved with GMP manufacture and
376 comprehensive phenotypic, genomic and transcriptomic characterisation of two *S*. Typhimurium
377 strains - D23580 and 4/74. The steps described here are comparable with those undertaken for
378 previous *Salmonella* challenge studies and serve as a benchmark for the type of testing required
379 for new challenge agent development. Phenotypic and genotypic characterisation demonstrated
380 that these two strains behaved as expected and no significant changes were introduced during
381 the manufacturing process, giving us confidence prior to their administration to healthy human
382 volunteers. We identified that both strains demonstrated improved survival in 0.9% saline
383 holding buffer compared with sodium bicarbonate, subsequently adapting our challenge
384 protocols in response to these data, modifying approaches used in previous *Salmonella*
385 challenge models. A novel aspect of this study is a more detailed characterisation of *Salmonella*
386 challenge stocks under conditions mimicking those encountered in the gastrointestinal tract
387 using a modified Rossett-Rice model. The addition of a transcriptomic assessment of virulence
388 gene expression offers a snapshot of the expected transcriptional state of the challenge agent as
389 it enters the stomach, providing new insights and assurances that key virulence factors are
390 unchanged in *Salmonella* following sodium bicarbonate pre-treatment which is a requirement of
391 accepted enteric challenge model protocols.

392 The strains used in this model were selected with careful consideration of the principles outlined
393 in **Table 1**. An ideal challenge model would incorporate multiple diverse strains to better reflect
394 pathogen diversity. Whilst this approach might be optimal, it is generally acknowledged that
395 practical limitations, including GMP manufacturing costs and regulatory complexity, often
396 preclude challenge studies from using more than one strain. Consequently, a detailed
397 characterisation of well-documented strains is considered the most feasible approach in most
398 circumstances. Provenance, traceable history from isolation, and prior comprehensive
399 characterisation provides greater assurance in the safety and reliability of this first-in-human
400 CHIM. *S.* Typhimurium was chosen as the preferred serovar for use in this model as it is
401 responsible for the greatest burden of iNTS in sSA and is supported by the most comprehensive
402 *in vitro* characterisation data.
403
404 Utilising two distinct strains of *S.* Typhimurium (ST19 4/74 and ST313 D23580) offers a unique
405 opportunity to evaluate the clinical, microbiological and immunological outcomes following
406 challenge in a healthy immune competent population. This platform facilitates direct
407 comparison between strains and therefore offers a deeper understanding of the possible impact
408 of genome degradation and human-adaptation observed in ST313, which may partly account for
409 its propensity to cause invasive disease in sSA. This also supports our approach to using wild-
410 type ST313 which harbours a distinct plasmid and prophage repertoire compared with ST19.
411 Whilst this strain is classified as multi-drug resistant, it remains fully sensitive to all clinically
412 relevant antibiotics used in the CHANTS study, ensuring participant safety.
413
414 In this study, challenge agent manufacture has been undertaken to GMP standard. Unlike some
415 other jurisdictions, the UK does not legally require challenge agents to be produced under GMP.
416 Approval from the UK Medicines and Healthcare products Regulatory Agency (MHRA) is only
417 necessary if the CHIM study involves an investigational medicinal product, such as if a vaccine

418 is administered alongside the challenge agent. Nevertheless, the MHRA guidance generally
419 recommends following GMP principles to ensure the safety and quality of the produced
420 challenge agent. Oversight primarily rests with the ethics committee and study sponsors [47,48].
421
422 *Salmonella* Typhimurium is often ascribed as one of the most comprehensively studied and
423 characterised pathogens and is a model organism for bacteriological research. Availability of
424 such a compendium of characteristic data was leveraged to facilitate the comprehensive
425 phenotypic and genomic characterisation undertaken in this study [49]. *In vitro* phenotypic
426 properties were confirmed to be consistent with published data including RDAR morphotype,
427 swimming motility and growth kinetics in a variety of nutrient media. Antimicrobial susceptibility
428 testing also confirmed activity of antibiotic agents selected for use within the CHANTS study.
429 Further, whole genome sequencing has confirmed genomic stability of both strains throughout
430 the manufacturing process and confirmed no genetic modifications which might alter expected
431 pathogenicity. Longitudinal sequencing of stored challenge agent stocks is also being
432 undertaken throughout the duration of the CHANTS study to confirm maintained genomic
433 stability.
434
435 Building on work from previous *Salmonella* challenge models, we have undertaken novel
436 assessments of the stability and viability of *Salmonella* in a range of pH conditions, holding
437 buffers, and in a simulated gastrointestinal environment, to model survival and phenotype at the
438 point of oral administration.
439
440 Our data indicated differences in the stability of *S.* Typhimurium in holding buffers compared with
441 previous *S.* Typhi and Paratyphi studies. Recent typhoidal *Salmonella* challenge studies
442 administer 2.1g/120ml of sodium bicarbonate buffer followed by the challenge agent in a
443 0.53g/30ml sodium bicarbonate holding buffer [37]. The data presented here indicate improved

444 bacterial survival in a 0.9% saline holding buffer compared with sodium bicarbonate. As a result,
445 the sample preparation SOP was updated so that the *S*. Typhimurium challenge agent is
446 administered in 30ml 0.9% saline, similar to the holding buffer used in recent *Shigella sonnei*
447 challenge studies [50]. In parallel, the bicarbonate buffer protocol was adjusted to account for
448 the reduction in sodium bicarbonate that would have been otherwise used as a holding buffer for
449 the 30ml *S*. Typhimurium inoculum. We now administer 2.6g/120ml sodium bicarbonate
450 solution, which provides the same total mass of bicarbonate ensuring equivalent buffering
451 capacity compared to previous *Salmonella* challenge study SOPs [37].
452
453 Bacterial cells remained stable in pH >3.5, stable for up to 2 hours in 0.9% saline holding buffer
454 and 1 hour in sodium bicarbonate and remained stable up to 60 minutes in a modified Rossett-
455 Rice model. These key findings provide assurance that viable bacterial cell counts remain stable
456 during the challenge agent preparation, transfer and administration protocols followed in the
457 CHANTS study. Furthermore, we have demonstrated that exposure to bicarbonate buffer does
458 not significantly alter *Salmonella* virulence factor gene expression. These findings taken together
459 provide assurance that bacterial cell concentration remains stable during challenge agent
460 preparation and transfer from the laboratory to clinic, and that gastric acid neutralisation with
461 bicarbonate pre-treatment does not impact pathogenicity at the point of oral administration,
462 thus ensuring close recapitulation of what likely occurs in natural exposure.
463
464 Whilst this study has provided a comprehensive characterisation of STm 4/74 and D23580 for
465 use in a first-in-human CHIM, there are limitations in our approach which should be considered.
466 Firstly, contemporary epidemiological genomic and surveillance studies highlight the important
467 contributions *S*. Enteritidis – and the emergence of *S*. Typhimurium ST313 sublineages 2.2 and
468 2.3 – have in iNTS disease burden [5,51]. Including these strains in our model could further
469 enhance generalisability to those isolates currently circulating in sSA, which account for a

470 significant proportion of iNTS disease. Furthermore, using recently harvested isolates – or
471 attenuated strains – could potentially compromise the rigorous ethical standards adhered to in
472 the development of CHIMs, as highlighted in **Table 1**. As such, strain selection was based upon
473 these core principles, with STm 4/74 and D23580 being selected as priority pathogens which
474 could provide the greatest translational data within the context of a CHIM.
475
476 In developing a nontyphoidal *Salmonella* (NTS) challenge model to mimic invasive disease, we
477 opted to develop two strains of *S*. Typhimurium for administration to volunteers, as the serovar
478 responsible for the majority of iNTS cases globally. Future plans include the development of a
479 *Salmonella* Enteritidis challenge model, which accounts for approximately one third of iNTS
480 cases worldwide. Like *S*. Typhimurium, *S*. Enteritidis strains associated with invasive disease in
481 low-income setting – such as SEn ST11 isolates from sSA [52] – have distinct genetic profiles
482 compared with those causing gastrointestinal disease in high-income regions. The strain
483 selection, development and manufacture of such a strain would follow principles similar to those
484 outlined here.
485
486 In summary, extensive characterisation of both *S*. Typhimurium D23580 and 4/74 has
487 experimentally confirmed predicted and known properties of these strains. These investigations
488 have significantly advanced the development of *Salmonella* human infection models and
489 support ongoing efforts to gain deeper understanding of host-pathogen interactions and immune
490 correlates of protection. Gaining such new understanding of iNTS infection can strengthen
491 ongoing efforts to support iNTS vaccine and therapeutic development. We aim to report CHANTS
492 study primary outcome data in the near future, with an outline of how these findings can be
493 translated to support ongoing efforts to combat iNTS disease in sSA.
494

# 4 MATERIALS AND METHODS

## 4.1 CHANTS Study Design

The CHANTS study detailed protocol has previously been described [53]. In brief, healthy UK participants aged 18-50 are randomised to receive oral challenge with one of two strains of *S.* Typhimurium (ST313 D23580 or ST19 4/74) in a double-blind, 1:1 randomisation protocol. A dose-escalation protocol is applied using the continual-reassessment method [54]. The primary objective is to determine the minimum infectious dose in colony forming units (CFU) for 60-75% of participants to develop systemic Salmonellosis (defined as bacteraemia and/or sustained fever T ≥38°C for ≥12 hours). Secondary objectives are to describe and compare the clinical, microbiological and immunological responses following challenge. Participants are monitored in a hospital environment for 7 days following challenge and are followed for 1 year to assess long-term safety.

## 4.2 Challenge Agent Strain Selection and Manufacture

STm ST313 D23580 [Ref 14677] and ST19 4/74 [Ref 14672] stocks were sourced from the UK Health Security Agency (UKHSA) National Collection of Type Cultures (NCTC) repository. Stocks were transferred to the Pilot Bioproduction Facility at Walter Reed Army Institute of Research (WRAIR), MD, USA. The challenge agent was prepared by inoculating a single colony of *Salmonella* Typhimurium strain 4/74 or D23580 from a Research Cell Bank ampoule onto Tryptic Soy Agar (TSA) plates. A selected colony was used to inoculate APS LB media in a baffled shake flask, which was incubated at 37 ± 1°C with shaking (200 ± 10 RPM) until the culture reached an OD600 of 2.0 ± 0.2. The culture was then mixed with 16% synthetic glycerol as a cryoprotectant, aliquoted into sterile cryovials, and flash-frozen for storage at ≤ -70°C in ultra-low temperature

521 freezers. Purity was ensured by streak plating, Gram staining, and dynamic environmental
522 monitoring compliant with Good Manufacturing Practices (GMP).
523
524 Colony counting was performed to assess the pre- and post-freeze viability of the challenge
525 strains. Two vials of each strain were thawed, and serial dilutions ($10^{-1}$ to $10^{-7}$) were prepared in
526 0.9% saline. Triplicate TSA plates were inoculated with 100µL of the $10^{-5}$ to $10^{-7}$ dilutions and
527 incubated at 37±1°C for 18-20 hours. Colony counts were conducted on plates containing 30–
528 300 colonies. Agglutination testing was conducted using the Sero-Quick Group kit to confirm
529 serogroup B antigen expression. Colonies were transferred to drops of Group B antiserum or
530 0.9% saline on a glass slide and observed for visible agglutination within 10 seconds.
531

532 **4.3 Microbial Limits Testing**
533

534 Manufactured challenge agent stocks underwent microbial limits testing for the presence of non-
535 sterile products, including specified organisms: Bile Tolerant Gram-Negative bacteria,
536 *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus spp., Clostridia*
537 *spp., Aspergillus spp.,* and *Candida spp*. Stocks were diluted in TSB diluent $10^{-3}$ to $10^{-8}$ and plated
538 on a range of organism specific selective media for microbial enumeration.
539

540 **4.4 Acid Sensitivity of *S.* Typhimurium in low pH buffers**
541

542 A pH range between 5 and 3.5 was used to evaluate the pH sensitivity of each STm challenge
543 strain. Test buffers were prepared in 50ml conical tubes with a mixture of simulated gastric fluid
544 (SGF; 7108-32, Lot 2301H18), sodium bicarbonate (Fisher S233-500, Lot 220180), and 0.9%
545 sterile saline (KD Medical RGF-3290, Lot 102021-0) to achieve the desired test pH. Cells were
546 added to each of the pH buffers at a concentration of $5.15 \times 10^5$ CFU/ml and held in a 37°C water
547 bath for pH adjusted samples or on ice for control samples for 15 minutes. After incubation the

548 buffers were neutralized with sodium bicarbonate and diluted with 0.9% saline to a final volume
549 of 32.1ml. Each test sample was plated in triplicate on 3 LB (Luria-Bertani) (Teknova, L7590)
550 plates (0.1ml per plate) and incubated overnight at 37°C prior to measuring mean CFU counts.
551

552 **4.5 *S.* Typhimurium Stability in 0.9% Saline Compared with Sodium Bicarbonate Buffer**
553

554 Stability of holding the challenge agents in bicarbonate buffer (2.6g/120ml) or 0.9% saline
555 solution was evaluated first with strain D23580 (ST313). The challenge agent was diluted in saline
556 or bicarbonate buffer to $5.15 \times 10^5$ CFU/ml and held on ice for 1, 2, or 3 hours prior to plating 0.1
557 mL on LB plates and incubating overnight at 37°C. Mean CFU counts were determined from two
558 technical replicates. Optimal pre-challenge holding conditions selected for D23580 were
559 confirmed with 4/74 (ST19) by diluting in 0.9% saline and holding for 1 hour on ice prior to plating
560 and counting as described above.
561

562 **4.6 *S.* Typhimurium Stability in a Modified Rossett-Rice Model**
563

564 Stability of the challenge strains was evaluated using a modified Rossett-Rice model (RR) at a 10-
565 fold reduced scale [55]. A sodium bicarbonate dose of 0.26g (representing a 2.6g human dose)
566 was evaluated and represents the formulation used in previous *Salmonella* Typhi and Paratyphi
567 models [20].
568

569 To determine the pH range expected in the RR model, a peristaltic pump (set at a rate of 0.4 ml
570 per minute) was used to add additional SGF throughout the experiment to mimic conditions in
571 the stomach. Additionally, a 1000ml beaker water bath was prepared and set to 37°C, and a
572 200ml beaker was placed inside. The stomach contents comprised 0.26g sodium bicarbonate in
573 12ml sterile water for injection (WFI; KD Medical RGF-3410, Lot 081720-04), 3ml of sterile 0.9%
574 saline, 0.1ml of 0.9% saline (substituted for cells), and 5ml of simulated gastric fluid (SGF; 7108-

575 32, Lot 2301H18). This resulted in a final buffer dose of 0.26g. The contents of the 50ml conical
576 tube were poured into the 200ml beaker and temperature and pH were monitored every minute
577 for a total of 70 minutes after an initial 5 minutes for acclamation.
578
579 To evaluate the challenge strain stability in the RR model, the RR was repeated with 0.1 ml of cells
580 diluted in 0.9% saline to a working concentration of $5.15x10^5$ CFU/ml. $1x10^9$ CFU/ml of each
581 strain mixed with glycerol was thawed on wet ice for 30 minutes. Once thawed, the cells were
582 diluted ten-fold three times in 15ml conical tubes with 0.9% saline to create a working stock of
583 cells at $5.15x10^5$ CFU/ml. Cells were held in 50ml conical tubes on ice for zero, one, and two
584 hours prior to testing in the Rossett-Rice model. The ability to recover bacteria by plating was
585 determined at 0, 30, 45, 60 minutes. Time points were plated on 4 technical replicate LB plates
586 (0.1ml per plate) and incubated overnight at 37°C prior to CFU counting.
587
588 To evaluate the challenge strain stability after transit into the small intestine, the RR model was
589 conducted as described above for 70 minutes, before samples were removed and added to
590 simulated intestinal fluid (Ricca 7109-32, Lot # 1412H21). 3.88ml samples of the RR ("gastric
591 fluid") were taken at 0, 30, 70min and mixed with 26.1ml of simulated intestinal fluid for a gastric
592 fluid to intestinal fluid ratio of $31cm^3/209cm^3$ [56]. Simulated intestinal fluid was filtered prior to
593 use and heated to 37°C. After addition of the gastric fluid to the simulated intestinal fluid the
594 solution was held at 37°C with stirring at 65 RPM. Samples of 0.1ml were plated and counted as
595 outlined above following overnight incubation.
596
597

598 ## 4.7 Phenotypic Characterisation
599

600 ### 4.7.1 Growth Characteristics
601

602 Growth curves for each strain were determined by culture in the Enzyscreen Growth Profiler in a
603 range of nutrient conditions. Lysogeny broth (LB) overnight cultures were diluted to optical
604 density $OD_{600}$ = 0.01 for inoculation into 96-well plates. Plates were incubated at 37°C for 24
605 hours with shaking at 220rpm, and measurements obtained every 30 minutes. The Growth
606 Profiler converts green pixel values of each well to optical density based on established
607 calibration curves for relevant media. Each growth curve was generated by the mean values of
608 six replicates. Growth curves for D23580 and 4/74 were assessed in LB media, minimal media
609 supplemented with 5% glucose, and minimal media supplemented with melibiose.
610

611 ### 4.7.2 Swimming Motility
612

613 Differential swimming motility between strains was assessed by spotting 3µl LB overnight culture
614 of D23580 and 4/74 onto 0.3% LB agar. Plates were left at room temperature for 30 minutes prior
615 to incubation at 37°C. The diameter of migration halos was assessed at 4 hours to determine the
616 relative bacterial swimming motility between strains.
617

618 ### 4.7.3 Red, Dry and Rough (RDAR) Morphotype
619

620 Red, dry and rough (RDAR) morphotype was assessed in NaCl-deficient LB plates supplemented
621 with 40µl/ml Congo red. 2µl of LB overnight culture was spotted onto plates and incubated at
622 room temperature for 72 hours prior to imaging colonial morphology using the ImageQuant 4000
623 biomolecular imager.
624

625 **4.7.4 Antibiotic Susceptibility**
626

627 Antibiotic susceptibility testing was undertaken by disc diffusion and broth microdilution in
628 accordance with EUCAST guidelines [57]. Disc diffusion was performed on Mueller-Hinton (MH)
629 agar with antibiotic discs applied and cultured overnight at 37°C. Interpretation as sensitive (S)
630 or resistant (R) was determined from zone diameter breakpoints produced by EUCAST.
631 Antibiotics tested include ampicillin (10µg), pefloxacin (5 µg), chloramphenicol (30 µg),
632 trimethoprim-sulphamethoxazole (25 µg), ciprofloxacin (5 µg), and ceftriaxone (30 µg).
633

634 Broth microdilution was undertaken using the automated BD Phoenix system. Minimum
635 inhibitory concentrations were measured in accordance with EUCAST guidelines to determine
636 susceptibility [36].
637

638 **4.8 Whole Genome Sequencing**
639

640 Genomic DNA was extracted using the ZYMO Research Quick-DNA Miniprep Plus Kit according
641 to the manufacturer's instructions. In summary, 1ml overnight LB culture was lysed then
642 incubated in a heat block at 55°C for 10 minutes. 420µl genomic binding buffer was added prior
643 to centrifugation and repeated wash with DNA buffer. 30µl DEPC water was added prior to final
644 centrifugation with resulting genomic DNA stored at -20°C. Illumina sequencing of extracted
645 genomic DNA was undertaken (SeqCenter, Pittsburgh, PA, USA), and sequence data was
646 analysed for mutations using SNIPPY software v4.6.0.
647

648 **4.9 Investigation of Bicarbonate Exposure on *S.* Typhimurium Virulence Factor Gene**
649 **Expression**
650

651 The impact of sodium bicarbonate exposure on virulence gene expression by STm D23580 and
652 4/74 was evaluated by transcriptomic profiling. RNA was extracted from D23580 and 4/74 after

653 incubation in water, and after 15- or 30-minutes incubation in 0.53g/30ml sodium bicarbonate
654 solution. RNA was extracted from STm strains using a modified TRIzol-based protocol. Overnight
655 cultures were grown in LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 37°C with shaking
656 (220 rpm). The cultures were diluted 1:1000 into fresh LB, grown to the desired $OD_{600}$, and mixed
657 with stop solution (5% phenol [pH 4.3], 95% ethanol) to stabilize RNA. Samples were centrifuged
658 at 3,220g for 10 minutes at 4°C, and bacterial pellets were resuspended and transferred to
659 microfuge tubes. The pellets were lysed in 1ml TRIzol reagent on ice, and RNA was extracted
660 using phase lock gel tubes. Following the addition of 400µl chloroform, samples were mixed,
661 incubated, and centrifuged to separate phases. The aqueous phase was recovered, and RNA was
662 precipitated with isopropanol. The resulting RNA pellets were washed with 70% ethanol, air-
663 dried, and dissolved in 20µL DEPC-treated, RNase-free water. RNA quality and concentration
664 were assessed using a Qubit fluorometer and Bioanalyzer (Agilent RNA 6000 Nano kit). RNA
665 samples were treated with DNase using the Invitrogen RNase-Free DNase Kit to remove residual
666 DNA. Libraries were prepared using Illumina's Stranded Total RNA Prep Ligation with Ribo-Zero
667 Plus kit, incorporating 10bp unique dual indices (UDI). Sequencing was performed on a NovaSeq
668 X Plus platform, generating paired end 150bp reads. Demultiplexing, quality control, and adapter
669 trimming were conducted using *bcl-convert* (v4.2.4). Sequence data was analysed in transcripts
670 per million (TPM) to assess relative gene expression.

# 5 REFERENCES


[1] Marchello CS, Fiorino F, Pettini E, Crump JA, Vacc-iNTS Consortium Collaborators. Incidence of non-typhoidal Salmonella invasive disease: A systematic review and meta-analysis. J Infect 2021;83:523–32. https://doi.org/10.1016/j.jinf.2021.06.029.

[2] GBD 2017 Non-Typhoidal Salmonella Invasive Disease Collaborators. The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis 2019;19:1312–24. https://doi.org/10.1016/S1473-3099(19)30418-9.

[3] Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet Lond Engl 2012;379:2489–99. https://doi.org/10.1016/S0140-6736(11)61752-2.

[4] Wilson CN, Chunga A, Masesa C, Denis B, Silungwe N, Bilima S, et al. Incidence of invasive non-typhoidal Salmonella in Blantyre, Malawi between January 2011-December 2019. Wellcome Open Res 2022;7:143. https://doi.org/10.12688/wellcomeopenres.17754.1.

[5] Van Puyvelde S, de Block T, Sridhar S, Bawn M, Kingsley RA, Ingelbeen B, et al. A genomic appraisal of invasive Salmonella Typhimurium and associated antibiotic resistance in sub-Saharan Africa. Nat Commun 2023;14:6392. https://doi.org/10.1038/s41467-023-41152-6.

[6] Pulford CV, Perez-Sepulveda BM, Canals R, Bevington JA, Bengtsson RJ, Wenner N, et al. Stepwise evolution of Salmonella Typhimurium ST313 causing bloodstream infection in Africa. Nat Microbiol 2021;6:327–38. https://doi.org/10.1038/s41564-020-00836-1.

[7] Chirwa EB, Dale H, Gordon MA, Ashton PM. What is the Source of Infections Causing Invasive Nontyphoidal *Salmonella* Disease? Open Forum Infect Dis 2023;10:ofad086. https://doi.org/10.1093/ofid/ofad086.

[8] MacLennan CA, Stanaway J, Grow S, Vannice K, Steele AD. *Salmonella* Combination Vaccines: Moving Beyond Typhoid. Open Forum Infect Dis 2023;10:S58–66. https://doi.org/10.1093/ofid/ofad041.

[9] Meloni E, Colucci AM, Micoli F, Sollai L, Gavini M, Saul A, et al. Simplified low-cost production of O-antigen from Salmonella Typhimurium Generalized Modules for Membrane Antigens (GMMA). J Biotechnol 2015;198:46–52. https://doi.org/10.1016/j.jbiotec.2015.01.020.

[10] Baliban SM, Curtis B, Toema D, Tennant SM, Levine MM, Pasetti MF, et al. Immunogenicity and efficacy following sequential parenterally-administered doses of Salmonella Enteritidis COPS:FliC glycoconjugates in infant and adult mice. PLoS Negl Trop Dis 2018;12:e0006522. https://doi.org/10.1371/journal.pntd.0006522.

[11] Choy RKM, Bourgeois AL, Ockenhouse CF, Walker RI, Sheets RL, Flores J. Controlled Human Infection Models To Accelerate Vaccine Development. Clin Microbiol Rev 2022;35:e0000821. https://doi.org/10.1128/cmr.00008-21.

[12] Jin C, Gibani MM, Moore M, Juel HB, Jones E, Meiring J, et al. Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of Salmonella Typhi: a randomised controlled, phase 2b trial. The Lancet 2017;390:2472–80. https://doi.org/10.1016/S0140-6736(17)32149-9.

[13] Talaat KR, Alaimo C, Martin P, Bourgeois AL, Dreyer AM, Kaminski RW, et al. Human challenge study with a Shigella bioconjugate vaccine: Analyses of clinical efficacy and correlate of protection. EBioMedicine 2021;66:103310. https://doi.org/10.1016/j.ebiom.2021.103310.

[14] Cohen MB. Human Challenge Studies for Cholera. Curr Top Microbiol Immunol 2022. https://doi.org/10.1007/82_2022_258.

[15] Smith C, Smith E, Chiu C, Hinton J, Perez Sepulveda B, Gordon M, et al. The Challenge Non-Typhoidal Salmonella (CHANTS) Consortium: Development of a non-typhoidal Salmonella controlled human infection model: Report from a consultation group workshop, 05 July 2022, London, UK. Wellcome Open Res 2023;8:111. https://doi.org/10.12688/wellcomeopenres.19012.2.

[16] Osowicki J, Azzopardi KI, McIntyre L, Rivera-Hernandez T, Ong C-LY, Baker C, et al. A Controlled Human Infection Model of Group A Streptococcus Pharyngitis: Which Strain and Why? mSphere 2019;4:e00647-18. https://doi.org/10.1128/mSphere.00647-18.

[17] Muhi S, Buultjens AH, Porter JL, Marshall JL, Doerflinger M, Pidot SJ, et al. Mycobacterium ulcerans challenge strain selection for a Buruli ulcer controlled human infection model. PLoS Negl Trop Dis 2024;18:e0011979. https://doi.org/10.1371/journal.pntd.0011979.

[18] La C. Considerations on the Principles of Development and Manufacturing Qaulities of Challenge Agents for use in Human Infection Models. Wellcome Trust; 2022.

[19] Canals R, Hammarlöf DL, Kröger C, Owen SV, Fong WY, Lacharme-Lora L, et al. Adding function to the genome of African Salmonella Typhimurium ST313 strain D23580. PLoS Biol 2019;17:e3000059. https://doi.org/10.1371/journal.pbio.3000059.

[20] Waddington CS, Darton TC, Jones C, Haworth K, Peters A, John T, et al. An Outpatient, Ambulant-Design, Controlled Human Infection Model Using Escalating Doses of Salmonella Typhi Challenge Delivered in Sodium Bicarbonate Solution. Clin Infect Dis 2014;58:1230–40. https://doi.org/10.1093/cid/ciu078.

[21] Cash R, Music S, Libonati JP, Craig JP, Pierce NF, Hornick R. Response of Man to Infection with Vibrio cholerae. II. Protection from Illness Afforded by Previous Disease and Vaccine. J Infect Dis 1974;130:325–33. https://doi.org/10.1105/tpc.104.024711.Briarty.

[22] Kotloff KL, Nataro JP, Losonsky GA, Wasserman SS, Hale TL, Taylor DN, et al. A modified Shigella volunteer challenge model in which the inoculum is administered with bicarbonate buffer: clinical experience and implications for Shigella infectivity. Vaccine 1995;13:1488–94. https://doi.org/10.1016/0264-410X(95)00102-7.

[23] Foster JW. The acid tolerance response of Salmonella typhimurium involves transient synthesis of key acid shock proteins. J Bacteriol 1993;175:1981–7. https://doi.org/10.1128/jb.175.7.1981-1987.1993.

[24] Brenneman KE, Willingham C, Kong W, Curtiss R, Roland KL. Low-pH Rescue of Acid-Sensitive Salmonella enterica Serovar Typhi Strains by a Rhamnose-Regulated Arginine Decarboxylase System. J Bacteriol 2013;195:3062–72. https://doi.org/10.1128/JB.00104-13.

[25] Soo Lee I, Lin J, Hall HK, Bearson B, Foster JW. The stationary-phase sigma factor $\sigma^S$ (RpoS) is required for a sustained acid tolerance response in virulent Salmonella typhimurium. Mol Microbiol 1995;17:155–67. https://doi.org/10.1111/j.1365-2958.1995.mmi_17010155.x.

[26] Van Den Abeele J, Rubbens J, Brouwers J, Augustijns P. The dynamic gastric environment and its impact on drug and formulation behaviour. Eur J Pharm Sci 2017;96:207–31. https://doi.org/10.1016/j.ejps.2016.08.060.

[27] Tzakri T, Rehenbrock L, Senekowitsch S, Rump A, Schick P, Krause J, et al. Determination of Gastric Water Emptying in Fasted and Fed State Conditions Using a Compression-Coated Tablet and Salivary Caffeine Kinetics. Pharmaceutics 2023;15:2584. https://doi.org/10.3390/pharmaceutics15112584.

[28] Grimm M, Koziolek M, Kühn J-P, Weitschies W. Interindividual and intraindividual variability of fasted state gastric fluid volume and gastric emptying of water. Eur J Pharm Biopharm 2018;127:309–17. https://doi.org/10.1016/j.ejpb.2018.03.002.

[29] Brock-Utne JG, Moshal MG, Downing JW, Spitaels JM, Stiebel R. Fasting volume and acidity of stomach contents associated with gastrointestinal symptoms. Anaesthesia 1977;32:749–52. https://doi.org/10.1111/j.1365-2044.1977.tb10075.x.

[30] Dressman JB, Berardi RR, Dermentzoglou LC, Russell TL, Schmaltz SP, Barnett JL, et al. Upper gastrointestinal (GI) pH in young, healthy men and women. Pharm Res 1990;7:756–61. https://doi.org/10.1023/a:1015827908309.
[31] Baron JH. The relationship between basal and maximum acid output in normal subjects and patients with duodenal ulcer. Clin Sci 1963;24:357–70.
[32] Carden S, Okoro C, Dougan G, Monack D. Non-typhoidal Salmonella Typhimurium ST313 isolates that cause bacteremia in humans stimulate less inflammasome activation than ST19 isolates associated with gastroenteritis. Pathog Dis 2015;73:ftu023. https://doi.org/10.1093/femspd/ftu023.
[33] Ramachandran G, Perkins DJ, Schmidlein PJ, Tulapurkar ME, Tennant SM. Invasive Salmonella Typhimurium ST313 with Naturally Attenuated Flagellin Elicits Reduced Inflammation and Replicates within Macrophages. PLoS Negl Trop Dis 2015;9:e3394. https://doi.org/10.1371/journal.pntd.0003394.
[34] Lokken KL, Walker GT, Tsolis RM. Disseminated infections with antibiotic-resistant non-typhoidal Salmonella strains: contributions of host and pathogen factors. Pathog Dis 2016;74:ftw103. https://doi.org/10.1093/femspd/ftw103.
[35] Singletary LA, Karlinsey JE, Libby SJ, Mooney JP, Lokken KL, Tsolis RM, et al. Loss of Multicellular Behavior in Epidemic African Nontyphoidal Salmonella enterica Serovar Typhimurium ST313 Strain D23580. mBio 2016;7:e02265-15. https://doi.org/10.1128/mBio.02265-15.
[36] The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 14.0, 2024 n.d. https://www.eucast.org/clinical_breakpoints (accessed December 31, 2024).
[37] Gibani MM, Jin C, Shrestha S, Moore M, Norman L, Voysey M, et al. Homologous and heterologous re-challenge with Salmonella Typhi and Salmonella Paratyphi A in a randomised controlled human infection model. PLoS Negl Trop Dis 2020;14:e0008783. https://doi.org/10.1371/journal.pntd.0008783.
[38] Gibani MM, Jones E, Barton A, Jin C, Meek J, Camara S, et al. Investigation of the role of typhoid toxin in acute typhoid fever in a human challenge model. Nat Med 2019;25:1082–8. https://doi.org/10.1038/s41591-019-0505-4.
[39] Tan A, Yang J, Tauschek M, Praszkier J, Robins-Browne RM. Autogenous Transcriptional Regulation of the *regA* Gene, Encoding an AraC-Like, Essential Virulence Regulator in *Citrobacter rodentium*. J Bacteriol 2011;193:1777–82. https://doi.org/10.1128/JB.01224-10.
[40] Abe H, Tatsuno I, Tobe T, Okutani A, Sasakawa C. Bicarbonate ion stimulates the expression of locus of enterocyte effacement-encoded genes in enterohemorrhagic Escherichia coli O157:H7. Infect Immun 2002;70:3500–9. https://doi.org/10.1128/IAI.70.7.3500-3509.2002.
[41] Yang J, Hart E, Tauschek M, Price GD, Hartland EL, Strugnell RA, et al. Bicarbonate-mediated transcriptional activation of divergent operons by the virulence regulatory protein, RegA, from *Citrobacter rodentium*. Mol Microbiol 2008;68:314–27. https://doi.org/10.1111/j.1365-2958.2008.06171.x.
[42] Avelino-Flores F, Soria-Bustos J, Saldaña-Ahuactzi Z, Martínez-Laguna Y, Yañez-Santos JA, Cedillo-Ramírez ML, et al. The Transcription of Flagella of Enteropathogenic Escherichia coli O127:H6 Is Activated in Response to Environmental and Nutritional Signals. Microorganisms 2022;10:792. https://doi.org/10.3390/microorganisms10040792.
[43] Mills DM, Bajaj V, Lee CA. A 40 kb chromosomal fragment encoding *Salmonella typhimurium* invasion genes is absent from the corresponding region of the *Escherichia coli* K-12 chromosome. Mol Microbiol 1995;15:749–59. https://doi.org/10.1111/j.1365-2958.1995.tb02382.x.

[44] Shea JE, Hensel M, Gleeson C, Holden DW. Identification of a virulence locus encoding a second type III secretion system in Salmonella typhimurium. Proc Natl Acad Sci 1996;93:2593–7. https://doi.org/10.1073/pnas.93.6.2593.
[45] Ilyas B, Tsai CN, Coombes BK. Evolution of Salmonella-Host Cell Interactions through a Dynamic Bacterial Genome. Front Cell Infect Microbiol 2017;7:428. https://doi.org/10.3389/fcimb.2017.00428.
[46] Das C, Mokashi C, Mande SS, Saini S. Dynamics and Control of Flagella Assembly in Salmonella typhimurium. Front Cell Infect Microbiol 2018;8:36. https://doi.org/10.3389/fcimb.2018.00036.
[47] The Academy of Medical Sciences. Controlled Human Infection Model Studies. Summary of a workshop held on 6 February 2018. 2018.
[48] Balasingam S, Meillon S, Chui C, Mann A, La C, Weller CL, et al. Human infection studies: Key considerations for challenge agent development and production. Wellcome Open Res 2022;7:140. https://doi.org/10.12688/wellcomeopenres.17869.1.
[49] SalCom (Salmonella Compendium, v2.0) n.d. https://bioinf.gen.tcd.ie/cgi-bin/salcom_v2.pl?_HL (accessed April 9, 2025).
[50] Frenck RW, Dickey M, Suvarnapunya AE, Chandrasekaran L, Kaminski RW, Clarkson KA, et al. Establishment of a Controlled Human Infection Model with a Lyophilized Strain of Shigella sonnei 53G. mSphere 2020;5:e00416-20. https://doi.org/10.1128/mSphere.00416-20.
[51] Kumwenda B, Canals R, Predeus AV, Zhu X, Kröger C, Pulford C, et al. Salmonella enterica serovar Typhimurium ST313 sublineage 2.2 has emerged in Malawi with a characteristic gene expression signature and a fitness advantage. microLife 2024;5:uqae005. https://doi.org/10.1093/femsml/uqae005.
[52] Feasey NA, Hadfield J, Keddy KH, Dallman TJ, Jacobs J, Deng X, et al. Distinct Salmonella Enteritidis lineages associated with enterocolitis in high-income settings and invasive disease in low-income settings. Nat Genet 2016;48:1211–7. https://doi.org/10.1038/ng.3644.
[53] Smith C, Smith E, Rydlova A, Varro R, Hinton JCD, Gordon MA, et al. Protocol for the challenge non-typhoidal *Salmonella* (CHANTS) study: a first-in-human, in-patient, double-blind, randomised, safety and dose-escalation controlled human infection model in the UK. BMJ Open 2024;14:e076477. https://doi.org/10.1136/bmjopen-2023-076477.
[54] O'Quigley J, Pepe M, Fisher L. Continual reassessment method: a practical design for phase 1 clinical trials in cancer. Biometrics 1990;46:33–48.
[55] Rossett NE, Rice ML. An In Vitro Evaluation of the Efficacy of the More Frequently Used Antacids with Particular Attention to Tablets. Gastroenterology 1954;26:490–5. https://doi.org/10.1016/S0016-5085(54)80029-1.
[56] Klinge MW, Sutter N, Mark EB, Haase A-M, Borghammer P, Schlageter V, et al. Gastric Emptying Time and Volume of the Small Intestine as Objective Markers in Patients With Symptoms of Diabetic Enteropathy. J Neurogastroenterol Motil 2021;27:390–9. https://doi.org/10.5056/jnm19195.
[57] Matuschek E, Brown DFJ, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect 2014;20:O255–66. https://doi.org/10.1111/1469-0691.12373.

# 6 SUPPORTING INFORMATION

**Ethics Statement**

London (Fulham) REC reference: 21/PR/0051

**Funding**

This work is funded by The Wellcome Trust (grant number 224029/Z/21/Z: awarded to principal investigator MMG). CS, GSC, and MMG are supported, in part, by the NIHR Imperial Biomedical Research Centre. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests**

The authors have declared that no competing interests exist.

**Author Contributions**

Conceptualisation – CS, MAG, GSC, RKMC, JCDH, MMG

Data Curation – CS, AB, XJ, JAW, NR, AES

Formal Analysis – CS, AB, XJ, LL

Funding Acquisition – MMG

Investigation – CS, AB, XJ, JAW, NR, AES, AR, RV, ZK, BPS

Project Administration – ES

Supervision – GSC, RKMC, JCDH, MMG

Writing – Original Draft Preparation – CS, JAW, XJ

Writing – Review and Editing – All Authors

**ORCID-IDs**

CS - http://orcid.org/0000-0001-7369-2034

XJ - https://orcid.org/0000-0002-1478-8500

MMG - http://orcid.org/0000-0003-1781-0053

RKMC - https://orcid.org/0000-0002-8441-4029

# 7 FIGURES

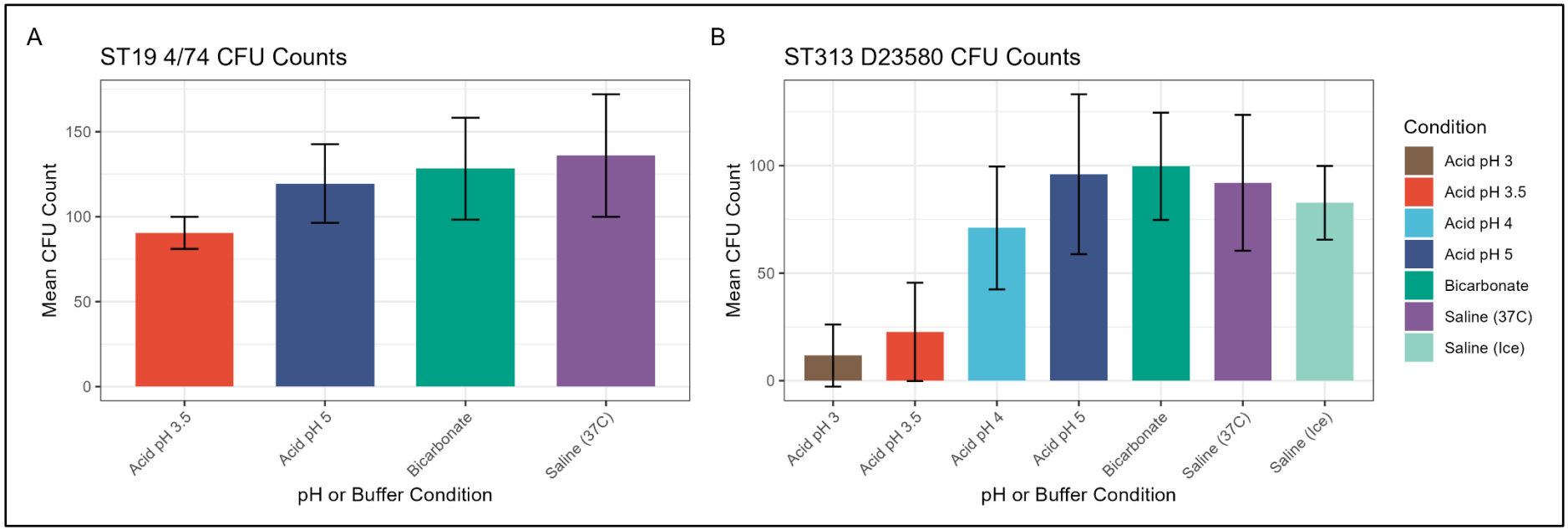


**Figure 1** – (A) STm 4/74 (ST19) and (B) D23580 (ST313) survival following incubation for 15 minutes in varying pH and temperature conditions. A broader range of conditions were tested for D23580 with the most relevant conditions being repeated with 4/74. Survival is stable in 0.9% saline, 2.6g/120ml bicarbonate buffer, and acid pH >4. A critical threshold of pH 3.5 was determined, with D23580 demonstrating greater sensitivity compared with 4/74. Mean CFU counts are calculated from three technical replicates.

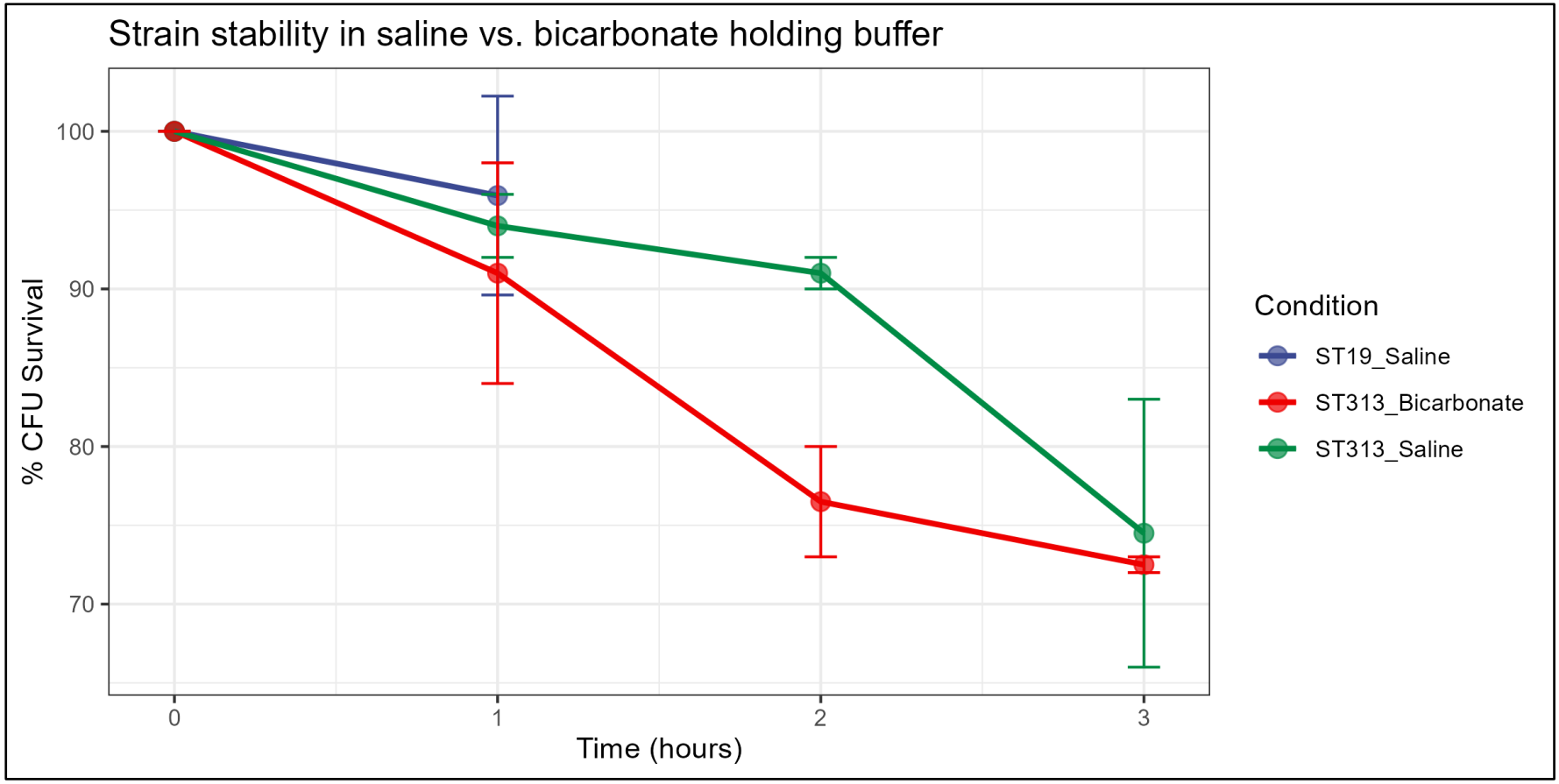


**Figure 2** – STm D23580 (ST313) and 4/74 (ST19) survival in 0.9% saline compared with 2.6g/120ml sodium bicarbonate buffer. 5.15 x $10^5$ cells were held in either 0.9% saline or sodium bicarbonate for up to 3 hours, with interval sampling every hour. D23580 was tested initially for full 3 hours, demonstrating marked reduction in survival in bicarbonate at 2 hours. 4/74 survival in 0.9% saline at 1 hour was confirmed to align with selected study protocols for acceptable upper time limits from preparation to participant administration. Percentage CFU survival taken as mean from 2 technical replicates per time point.

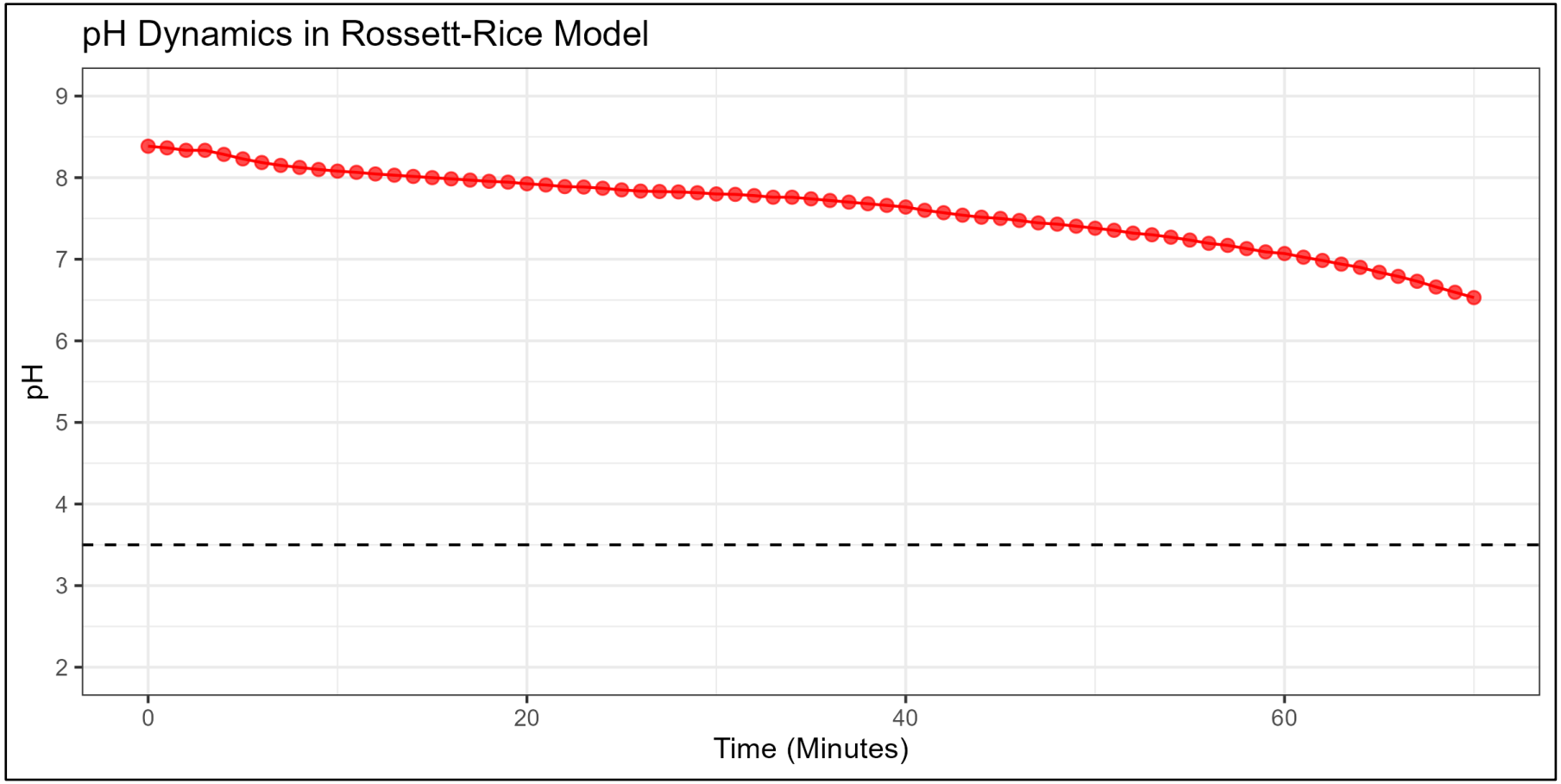


**Figure 3** – Simulated gastric fluid pH in modified Rossett-Ross *in vitro* system following administration of 0.26g bicarbonate. A peristaltic pump (set at a rate of 0.4 ml per minute) was used to add additional SGF throughout the experiment to mimic conditions in the stomach, with pH monitoring every minute. pH >6.5 is maintained to 70 minutes, maintaining pH above the previously identified critical survival threshold of 3.5.

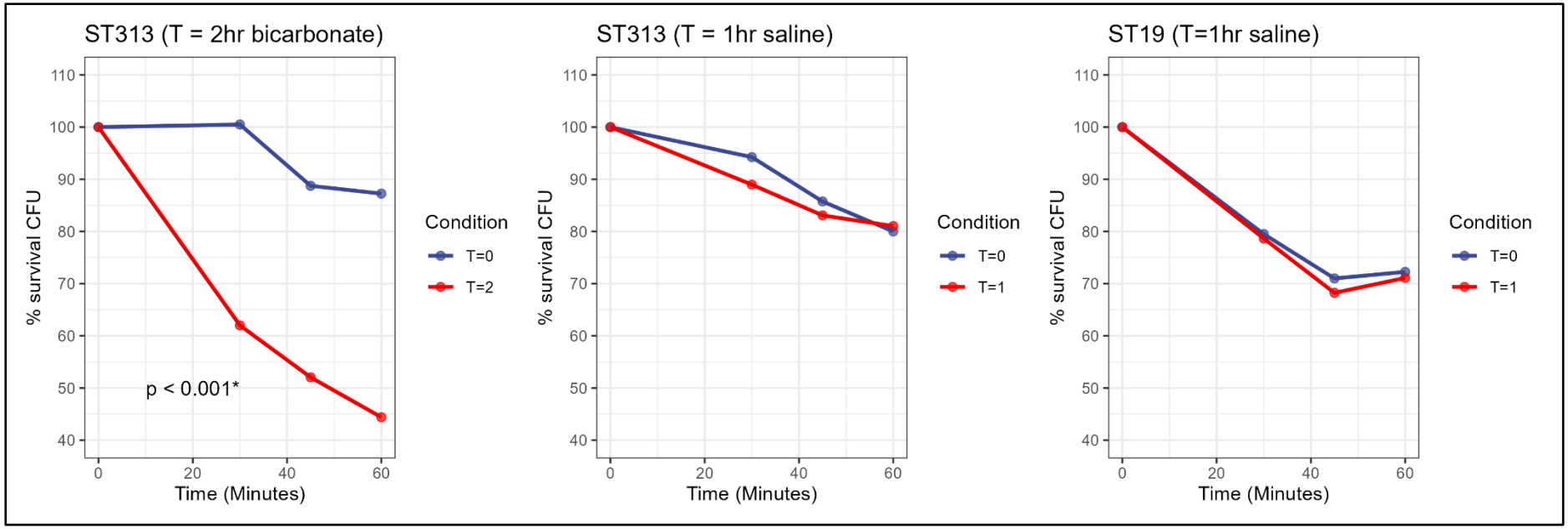


**Figure 4** – STm D23580 (ST313) and 4/74 (ST19) survival in the modified Rossett-Rice *in vitro* system, simulating bacterial transit through the upper gastrointestinal tract. Incubation conditions tested prior to addition to the RR model included 0, 1 or 2 hours hold on ice in either 0.9% saline or bicarbonate buffer. D23580 demonstrated marked reduction in survival following 2-hour hold in bicarbonate ($p<0.001$). Both D23580 and 4/74 demonstrated comparable rates survival following 0 or 1 hour hold on ice in 0.9% saline. Mean and standard deviation CFU survival calculated from 4 technical replicates per time point.

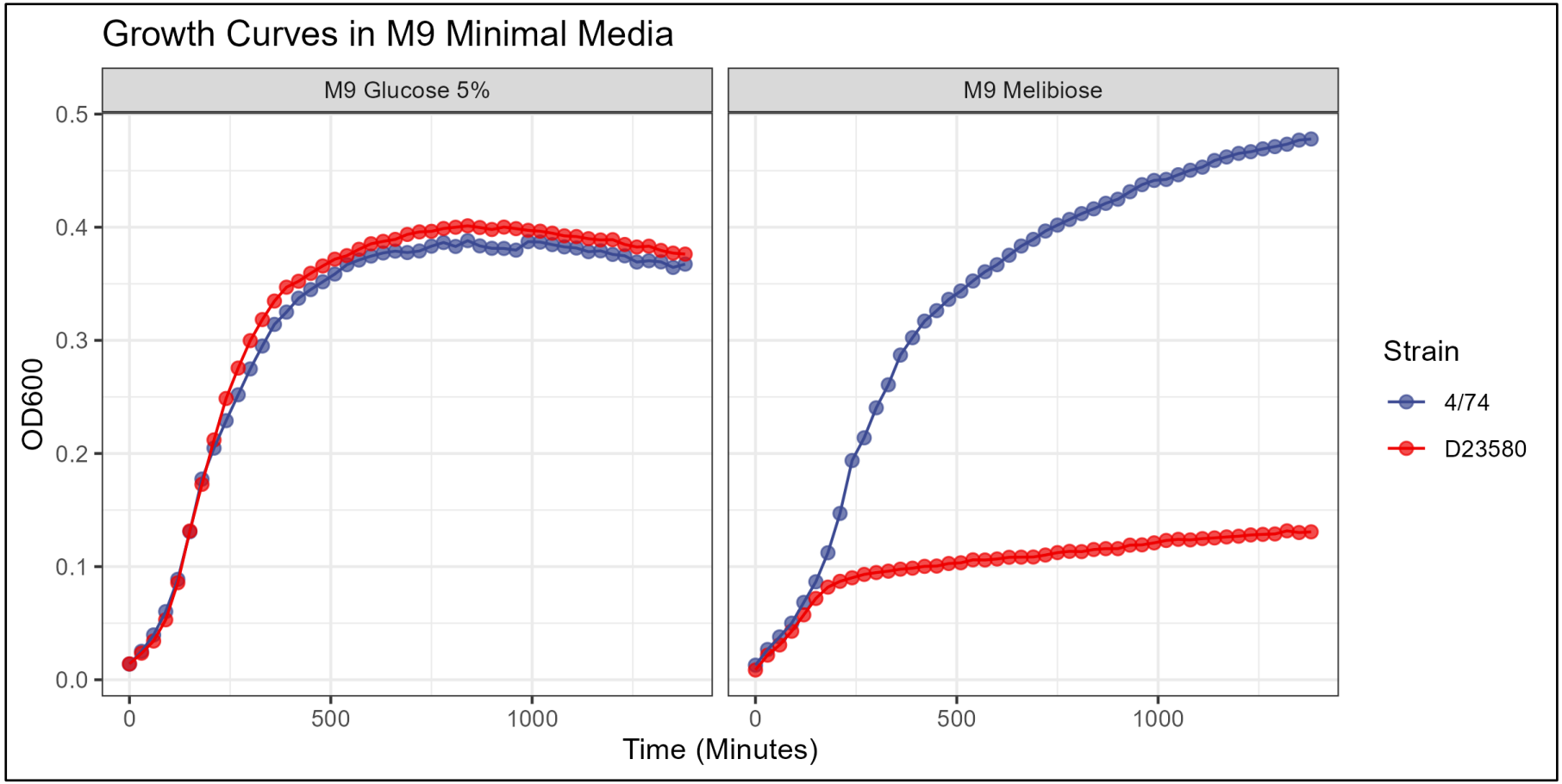


**Figure 5** – Growth curves for STm ST19 4/74 and ST313 D23580 in M9 minimal media supplemented with 5% glucose (left) and melibiose (right). Analysed by Enzyscreen Growth Profiler over 24 hours, starting OD600 = 0.01. Comparable growth patterns are observed in 5% glucose, whilst growth of D23580 is inhibited in melibiose. Mean OD600 values at each time point calculated from six technical replicates.

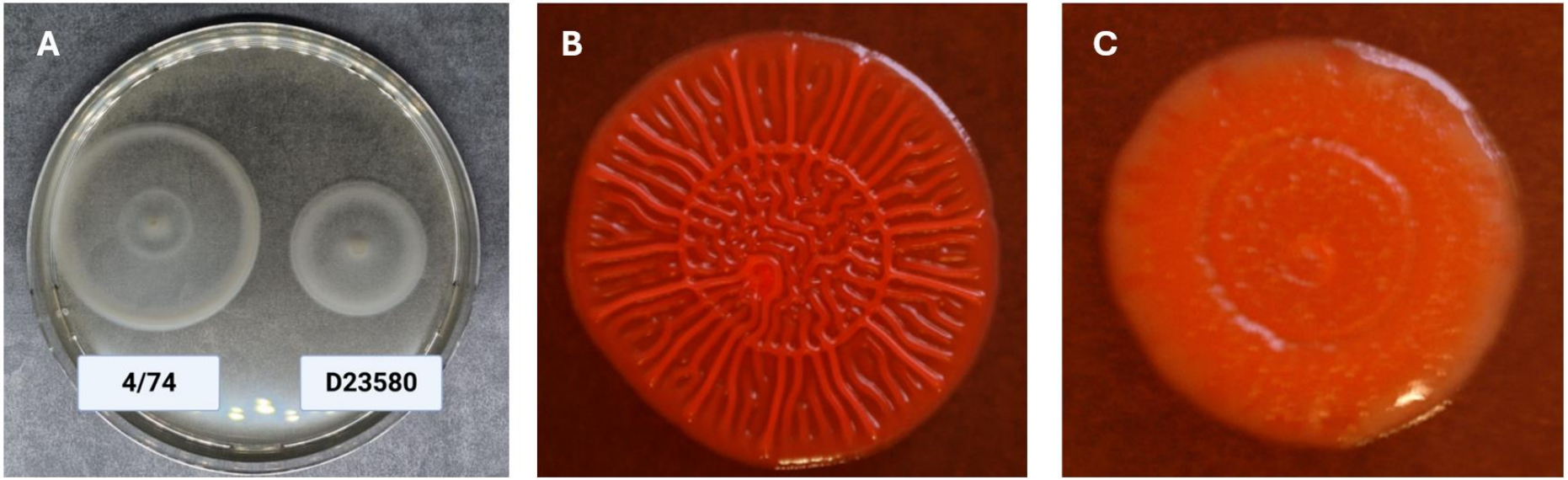


**Figure 6** – (A) Swimming motility of STm 4/74 (left) and D23580 (right) in 0.3% LB agar. Photographed after 4 hours incubation at 37C. The larger halo diameter of 4/74 illustrates increased swimming motility (and flagellar expression) of 4/74 as compared with D23580. (B) Red, dry and rough (RDAR) morphotype colonies of STm 4/74 and (C) D23580 after 72 hours incubation at room temperature on NaCl-deficient LB agar supplemented with Congo red. Imaged using the ImageQuant 4000 biomolecular imager.

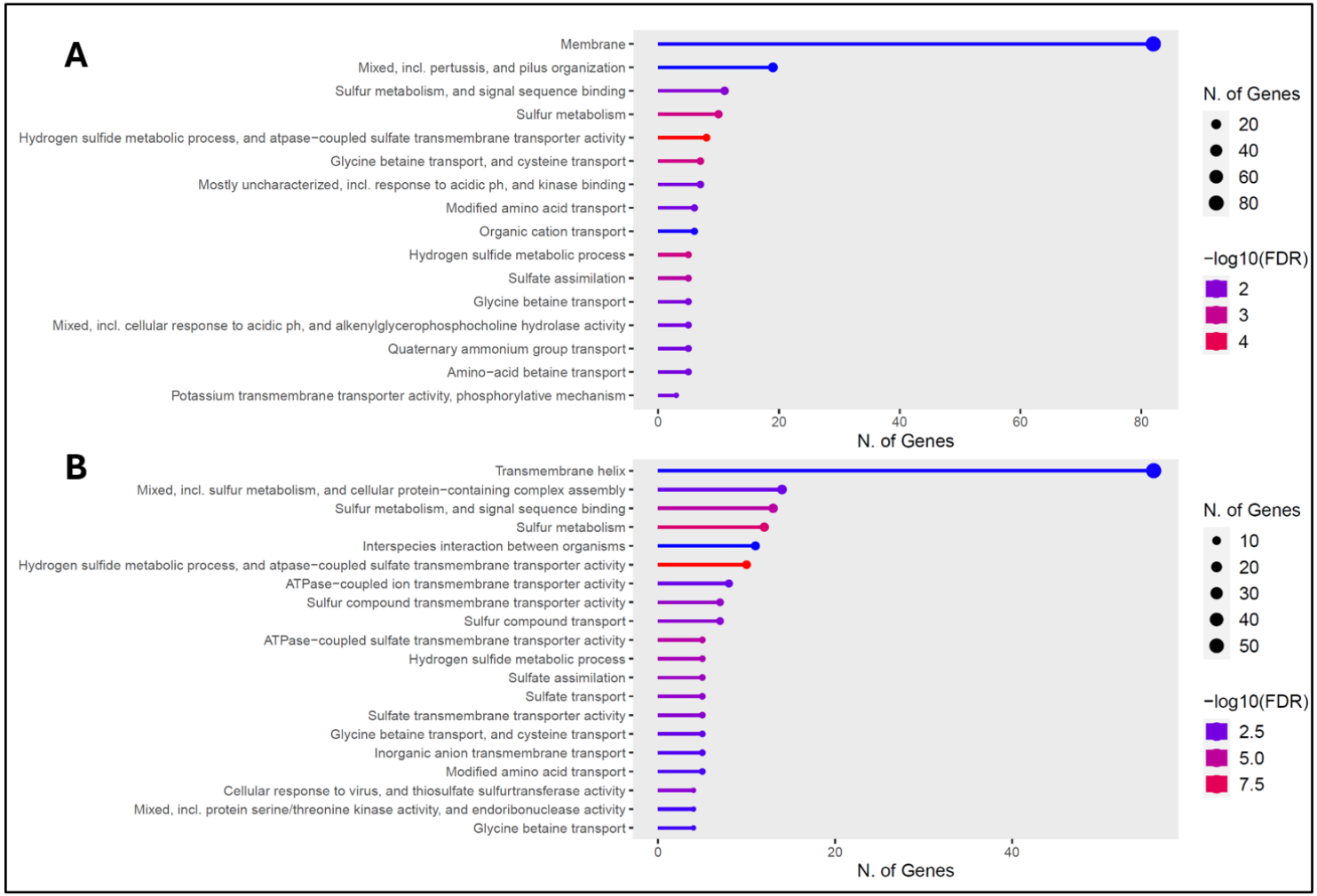


**Figure 7** – (A) STm 4/74 (ST19) and (B) D23580 (ST313) ontology of significantly differentially expressed genes pre- and post-bicarbonate exposure. Membrane and transmembrane helix associated genes are affected with greatest frequency, however none are associated with established SPI-1, SPI-2 or flagellar virulence genes.