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

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Abstract

Background

Invasive non-typhoidal *Salmonella* (iNTS) disease remains a major public health challenge in sub-Saharan Africa. *Salmonella* Typhimurium is responsible for the majority of cases, with specific lineages being associated with increased risk of bloodstream infection. We have recently developed a *Salmonella* Typhimurium 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 the two *Salmonella* Typhimurium strains used in the first-in-human NTS CHIM.

Methods

Two Salmonella Typhimurium strains, 4/74 (ST19) and D23580 (ST313), were selected from the UK Health Security Agency National Collection of Type Cultures and manufactured under Good Manufacturing Practice (GMP) conditions. Challenge agent stocks underwent microbial limits testing, viability and stability assessments, and phenotypic characterisation including growth kinetics, motility, acid sensitivity, and antibiotic susceptibility. Whole-genome sequencing was performed to confirm genetic stability post-manufacture. The effect of pre-challenge handling conditions was assessed in saline and sodium bicarbonate buffers, and bacterial survival was evaluated under simulated gastric and intestinal conditions using a modified Rossett–Rice model. Transcriptomic profiling was undertaken to determine whether sodium bicarbonate exposure altered expression of key virulence genes.

Results

Both strains retained their expected phenotypic characteristics, including reduced motility and melibiose utilisation in D23580. GMP stocks remained pure, viable, and stable post-manufacture, with no unexpected genomic mutations detected. Both strains were susceptible to clinically relevant antibiotics used in the study. Survival was maintained in neutral and mildly acidic conditions, with significant reduction below pH 3.5. Stability was preserved for up to one hour in saline buffer and during simulated gastric transit in the Rossett-Rice model. Transcriptomic analysis showed no significant changes in *Salmonella* pathogenicity island-1 or 2, or flagellar gene expression following sodium bicarbonate exposure

Conclusions

These findings confirm that the *Salmonella* Typhimurium 4/74 and D23580 strains retained genomic and phenotypic integrity post-manufacture. The resulting challenge stocks provide the foundation for an ongoing NTS CHIM that aims to advance understanding of NTS and iNTS pathogenesis and support candidate vaccine testing.

Keywords: *Salmonella*; *Salmonella* Typhimurium; Non-typhoidal *Salmonella*; Human Challenge Model; Controlled Human Infection Model; Vaccines.

43 # 1 Introduction

44

45 Invasive non-typhoidal *Salmonella* (iNTS) is a major cause of community-acquired bloodstream
46 infection in sub-Saharan Africa (sSA) and is associated with significant morbidity and mortality
47 [1]. iNTS disease was estimated to have caused 535,000 cases and 77,500 deaths in 2017 [2].
48 The greatest burden is observed in children under 5 years, with recognised risk factors including
49 anaemia, malnutrition, and recent malaria infection [3]. Immunocompromised adults with HIV
50 are also susceptible; however, incidence of HIV-associated infection has been declining with
51 improved access to effective antiretroviral therapy [4].

52

53 *Salmonella* Typhimurium (STm) is the most commonly isolated serovar in iNTS bloodstream
54 infections, followed by *Salmonella* Enteritidis (SEn) [5]. STm multi-locus sequence type (ST)313
55 has been identified as the dominant circulating pathovar in sSA and is associated with multi-drug
56 resistance to amoxicillin, chloramphenicol and co-trimoxazole [5,6]. Additionally, the dominant
57 circulating *S*. Typhimurium strains responsible for the majority of infections have been shown to
58 have undergone evolutionary changes, characterised by genomic degradation and pseudogene
59 formation, which are postulated to represent host adaptation towards an invasive phenotype [6].
60 The relative contributions of host susceptibility and host-adapted bacterial factors in iNTS are
61 incompletely understood.

62

63 In addition to challenges posed by antimicrobial resistance (AMR), public health interventions to
64 control iNTS disease burden are hampered by an incomplete understanding of transmission
65 reservoirs and a lack of preventative vaccines [7]. Several promising vaccine candidates are in
66 early-stage human development, including core and O-polysaccharide (COPS)/FliC conjugate
67 vaccines and those utilising the Generalised Modules for Membrane Antigens (GMMA) platform

68 [8–11]. The leading candidate vaccines are likely to progress as multivalent formulations
69 targeting both STm and SEn, potentially alongside antigenic targets for typhoidal *Salmonella*.
70
71 Controlled human infection models (CHIMs) can provide unique insights into mechanisms of
72 disease pathogenesis and elucidate immune correlates of protection [12]. In some
73 circumstances, CHIMs can also provide clinically relevant platforms for candidate vaccine
74 efficacy studies, supporting licensure decisions [13]. This has been particularly true for enteric
75 pathogens, where CHIMs have played a central role in accelerating development of vaccines
76 against *S*. Typhi and *V. cholerae* [14–16].
77
78 We have recently established a first-in-human NTS CHIM. The Challenge Non-typhoidal
79 *Salmonella* (CHANTS) model aims to characterise the clinical and microbiological responses of
80 healthy volunteers to oral challenge with *S*. Typhimurium, describe putative immune correlates
81 of protection, and provide a platform for future NTS vaccine efficacy assessments. A consortium
82 of experts in the fields of *Salmonella* biology, epidemiology, vaccine development and human
83 challenge models have contributed to the study design [17].
84
85 Several considerations relating to bacterial challenge strain selection, manufacture and
86 characterisation are central to the safe and ethical conduct of controlled human infection
87 models. The approach to strain selection has been described in detail for other bacterial human
88 challenge models [18,19]. Whilst some pathogen-specific considerations may apply, some
89 general principles are outlined in **Table 1**. To maximise the utility of a disease model, challenge
90 strains should aim to be as a minimum: i) phylogenetically representative of contemporary
91 disease-causing isolates; ii) susceptible to commonly used antibiotics; iii) have a traceable
92 history from isolation; iv) possess a representative repertoire of virulence factors and v) be
93 associated with a relevant disease phenotype for vaccine efficacy assessment. Here we describe

94 the strategy used to select the most relevant strains for use in a novel NTS CHIM. We provide
95 comprehensive phenotypic and genomic characterisation of selected strains and detail the
96 testing protocols associated with manufacture to GMP standards. We go on to explore the effect
97 of sodium bicarbonate exposure on *S*. Typhimurium virulence gene expression, in an attempt to
98 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 • 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. • 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. • 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 • 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 • Production should be within laboratory facilities with appropriate Biosafety containment level suitable for the specific CA • 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 • Purity: CA should be free from other agents of human infection (viruses, bacteria, fungi), determined by microbial limits testing • 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 • 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 • 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 • 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 [20].

2 Materials and Methods

2.1 CHANTS Study Design

The CHANTS study detailed protocol has previously been described [21]. In brief, healthy UK participants aged 18-50 are randomised to receive oral challenge with one of two strains of *S.* Typhimurium - D23580 (ST313) or 4/74 (ST19) – in a double-blind, 1:1 randomisation protocol. A dose-escalation protocol is applied using the continual-reassessment method [22]. 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 (oral temperature ≥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.

2.2 Challenge Agent Strain Selection and Manufacture

STm D23580 and 4/74 stocks were sourced from the UK Health Security Agency National Collection of Type Cultures repository. Stocks were transferred to the Pilot Bioproduction Facility at Walter Reed Army Institute of Research, MD, USA for manufacture under Good Manufacturing Practice (GMP) conditions. Each challenge agent was prepared by inoculating a single colony of *Salmonella* Typhimurium from a research cell bank ampoule onto Tryptic Soy Agar (TSA) plates. A single 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 freezers. Purity

128 was ensured by streak plating, Gram staining, and dynamic environmental monitoring compliant
129 with GMP standards.
130
131 Colony counting was performed to assess the pre- and post-freeze viability of the challenge
132 strains. Two vials of each strain were thawed, and serial dilutions (10^{-1} to 10^{-7}) were prepared in
133 0.9% saline. Triplicate TSA plates were inoculated with 100µL of the 10^{-5} to 10^{-7} dilutions and
134 incubated at 37±1°C for 18-20 hours. Colony counts were conducted on plates containing 30–
135 300 colonies. Agglutination testing was conducted using the Sero-Quick Group kit to confirm
136 serogroup B antigen expression. Colonies were transferred to drops of Group B antiserum or
137 0.9% saline on a glass slide and observed for visible agglutination within 10 seconds.
138

139 **2.3 Microbial Limits Testing**
140

141 Manufactured challenge agent stocks underwent microbial limits testing for the presence of non-
142 sterile products, including specified organisms: Bile Tolerant Gram-Negative bacteria,
143 *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus spp., Clostridia*
144 *spp., Aspergillus spp.,* and *Candida spp*. Stocks were diluted in TSB diluent 10^{-3} to 10^{-8} and plated
145 on a range of organism specific selective media for microbial enumeration.
146

147 **2.4 Acid Sensitivity of *S.* Typhimurium in low pH buffers**
148

149 A pH range from pH 5 to pH 3.5 was used to evaluate acid sensitivity of each challenge strain.
150 Test buffers were prepared in 50ml conical tubes with a mixture of simulated gastric fluid (SGF;
151 7108-32, Lot 2301H18), sodium bicarbonate (Fisher S233-500, Lot 220180), and 0.9% sterile
152 saline (KD Medical RGF-3290, Lot 102021-0) to achieve the desired test pH. Cells were added to
153 each of the pH buffers at a concentration of 5.15×10^5 CFU/ml and held in a 37°C water bath for
154 pH adjusted samples or on ice for control samples for 15 minutes. After incubation the buffers

155 were neutralized with sodium bicarbonate and diluted with 0.9% saline to a final volume of
156 32.1ml. Each test sample was plated in triplicate on LB (Teknova, L7590) media (0.1ml per plate)
157 and incubated overnight at 37°C prior to measuring mean CFU counts (n=3).
158

159 **2.5 *S.* Typhimurium Stability in 0.9% Saline Compared with Sodium Bicarbonate Buffer**
160

161 Stability of holding the challenge agents in bicarbonate buffer (2.6g/120ml) or 0.9% saline
162 solution was evaluated first with D23580. The challenge agent was diluted in saline or
163 bicarbonate buffer to 5.15×10^5 CFU/ml and held on ice for up to 3 hours. 0.1 mL of solution was
164 sampled at hourly intervals and plated on LB prior to overnight incubation at 37°C. Mean CFU
165 counts were determined from three replicate LB plates per time point, with the experiment being
166 performed in duplicate. Upon identification of optimal pre-challenge holding conditions for
167 D23580 (0.9% saline; 1-hour hold), the relevant conditions were tested and confirmed with 4/74
168 by diluting in 0.9% saline and holding for 1 hour on ice prior to plating and counting as described
169 above.
170

171 **2.6 Simulated Gastrointestinal pH Dynamics in a Modified Rossett-Rice Model**
172

173 Stability of the challenge strains was evaluated using a modified Rossett-Rice model (RR) at a 10-
174 fold reduced scale [23]. A sodium bicarbonate dose of 0.26g (representing a 2.6g human dose)
175 was evaluated and represents the formulation used in previous *Salmonella* Typhi and Paratyphi
176 challenge models [24].
177

178 To first determine the pH range expected in the RR model, a peristaltic pump (set at a rate of 0.4
179 ml per minute) was used to add additional SGF throughout the experiment to mimic conditions
180 in the stomach. Additionally, a 1000ml beaker water bath was prepared and set to 37°C with a
181 200ml beaker placed inside. The stomach contents comprised 0.26g sodium bicarbonate in

182 12ml sterile water for injection (WFI; KD Medical RGF-3410, Lot 081720-04), 3ml of sterile 0.9%
183 saline, 0.1ml of 0.9% saline (substituted for cells), and 5ml of simulated gastric fluid (SGF; 7108-
184 32, Lot 2301H18). This resulted in a final buffer dose of 0.26g. The contents of the 50ml conical
185 tube were poured into the 200ml beaker and temperature and pH were monitored every minute
186 for a total of 70 minutes after an initial 5 minutes for acclimation.
187

188 **2.7 *S.* Typhimurium Stability in a Modified Rossett-Rice Model**
189

190 To evaluate challenge strain stability in the RR model, the RR was repeated with 0.1 ml of cells
191 diluted in 0.9% saline or 0.26g bicarbonate buffer to a working concentration of 5.15×10^5 CFU/ml.
192 Cells were held in 50ml conical tubes on ice for zero, one, and two hours prior to testing in the
193 Rossett-Rice model. The ability to recover bacteria was determined by plating at 0, 30, 45, and
194 60 minutes. Time points were plated on four replicate LB plates (0.1ml per plate) and incubated
195 overnight at 37°C prior to CFU counting.
196

197 To evaluate the challenge strain stability after transit into the small intestine, the RR model was
198 conducted as described above for 70 minutes, before samples were removed and added to
199 simulated intestinal fluid (Ricca 7109-32, Lot # 1412H21). 3.88ml samples of the RR ("gastric
200 fluid") were taken at 0, 30, 70min and mixed with 26.1 ml of simulated intestinal fluid for a gastric
201 fluid to intestinal fluid ratio of $31cm^3/209cm^3$ [25]. Simulated intestinal fluid was filtered prior to
202 use and heated to 37°C. After addition of the gastric fluid to the simulated intestinal fluid the
203 solution was held at 37°C with stirring at 65 RPM. Samples of 0.1ml were plated and counted as
204 outlined above following overnight incubation.

205 ## 2.8 Phenotypic Characterisation
206

207 ### 2.8.1 Growth Characteristics
208

209 Growth curves for each strain were determined by culture in the Enzyscreen Growth Profiler in a
210 range of nutrient conditions. LB overnight cultures were diluted to optical density OD_{600} = 0.01 for
211 inoculation into 96-well plates. Plates were incubated at 37°C for 24 hours with shaking at
212 220rpm, and measurements obtained every 30 minutes. The Growth Profiler converts green pixel
213 values of each well to optical density based on established calibration curves for relevant media.
214 Each growth curve was generated by the mean values of six replicates. Growth curves for D23580
215 and 4/74 were assessed in LB media, M9 minimal media supplemented with 5% glucose, and M9
216 minimal media supplemented with melibiose.
217

218 ### 2.8.2 Swimming Motility
219

220 Differential swimming motility between strains was assessed by spotting 3 µl LB overnight culture
221 of D23580 and 4/74 onto 0.3% LB agar. Plates were left at room temperature for 30 minutes prior
222 to incubation at 37°C. The diameter of migration halos was assessed at 4 hours to determine the
223 relative bacterial swimming motility between strains.
224

225 ### 2.8.3 Red, Dry and Rough (RDAR) Morphotype
226

227 Red, dry and rough (RDAR) morphotype was assessed in NaCl-deficient LB plates supplemented
228 with 40µl/ml Congo red. 2µl of LB overnight culture was spotted onto plates and incubated at
229 room temperature for 72 hours prior to imaging colonial morphology using the ImageQuant 4000
230 biomolecular imager.
231

232 **2.8.4 Antibiotic Susceptibility**
233

234 Antibiotic susceptibility testing was undertaken by disc diffusion and broth microdilution in
235 accordance with EUCAST guidelines [26]. Disc diffusion was performed on Mueller-Hinton (MH)
236 agar with antibiotic discs applied and cultured overnight at 37°C. Interpretation as sensitive (S)
237 or resistant (R) was determined from zone diameter breakpoints produced by EUCAST.
238 Antibiotics tested include ampicillin (10µg), pefloxacin (5 µg), chloramphenicol (30 µg),
239 trimethoprim-sulphamethoxazole (25 µg), ciprofloxacin (5 µg), and ceftriaxone (30 µg).
240

241 Broth microdilution was undertaken using the automated BD Phoenix system. Minimum
242 inhibitory concentrations were measured in accordance with EUCAST guidelines to determine
243 susceptibility [27]. All antibiotic susceptibility testing was performed in accredited diagnostic
244 facilities at North-West London Pathology, Imperial College Healthcare NHS Trust.
245

246 **2.9 Whole Genome Sequencing**
247

248 Genomic DNA was extracted using the ZYMO Research Quick-DNA Miniprep Plus Kit according
249 to the manufacturer's instructions. In summary, 1ml overnight LB culture was lysed then
250 incubated in a heat block at 55°C for 10 minutes. 420µl genomic binding buffer was added prior
251 to centrifugation and repeated wash with DNA buffer. 30µl DEPC water was added prior to final
252 centrifugation with resulting genomic DNA stored at -20°C. Illumina sequencing of extracted
253 genomic DNA was undertaken (SeqCenter, Pittsburgh, PA, USA), and sequence data was
254 analysed for mutations using SNIPPY software v4.6.0.
255
256

257 **2.10 Investigation of Bicarbonate Exposure on *S.* Typhimurium Virulence Factor Gene**
258 **Expression**
259

260 The impact of sodium bicarbonate exposure on virulence gene expression by D23580 and 4/74
261 was evaluated by transcriptomic profiling. RNA was extracted from D23580 and 4/74 after
262 incubation in either 0-minutes or 30-minutes incubation in 0.53g/30ml sodium bicarbonate
263 solution. RNA was extracted from STm strains using a modified TRIzol-based protocol. Overnight
264 cultures were grown in LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) at 37°C with shaking
265 (220 rpm). Cultures were diluted 1:1000 into fresh LB, grown to the desired OD_{600} = 2.0. Cells were
266 centrifuged then mixed with stop solution (5% phenol [pH 4.3], 95% ethanol) to stabilize RNA
267 either immediately (0-minutes) or after 30-minutes incubation in sodium bicarbonate solution.
268 Samples were centrifuged at 3,220g for 10 minutes at 4°C, and bacterial pellets were
269 resuspended and transferred to microfuge tubes. The pellets were lysed in 1 ml TRIzol reagent on
270 ice, and RNA was extracted using phase lock gel tubes. Following the addition of 400µl
271 chloroform, samples were mixed, incubated, and centrifuged to separate phases. The aqueous
272 phase was recovered, and RNA was precipitated with isopropanol. The resulting RNA pellets
273 were washed with 70% ethanol, air-dried, and dissolved in 20µL DEPC-treated, RNase-free
274 water. RNA quality and concentration were assessed using a Qubit fluorometer and Bioanalyzer
275 (Agilent RNA 6000 Nano kit). RNA samples were treated with DNase using the Invitrogen RNase-
276 Free DNase Kit to remove residual DNA. Libraries were prepared using Illumina's Stranded Total
277 RNA Prep Ligation with Ribo-Zero Plus kit, incorporating 10bp unique dual indices (UDI).
278 Sequencing was performed on a NovaSeq X Plus platform, generating paired end 150bp reads.
279 Demultiplexing, quality control, and adapter trimming were conducted using *bcl-convert*
280 (v4.2.4). Sequence data was analysed in transcripts per million (TPM) to assess relative gene
281 expression.

3 Results

3.1 Challenge Strain Selection

Strain selection for use in the CHANTS study has been conducted in accordance with the principles and considerations outlined in **Table 1**. The CHANTS study uses two distinct strains of *S*. Typhimurium which are considered representative of both invasive (iNTS) and diarrhoeal (dNTS) disease-causing isolates. D23580 belongs to the dominant ST313 sequence type causing invasive disease in sSA, whilst 4/74 (ST19) is considered an archetypal strain representative of globally distributed infections causing enterocolitis [5]. In addition to their epidemiological relevance, D23580 and 4/74 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 [28,29]. Both strains have been deposited in the UK Health Security Agency National Collection of Type Cultures and have been sourced from this repository for the purposes of this study.

The final selection of these specific strains was informed by expert consultation and ultimately based on their status as well-characterised, archetypal laboratory strains representing the two major lineages of *Salmonella* Typhimurium [17]. These choices enabled us to leverage extensive microbiological, genomic and pathogenesis data, allowing presentation of strain characteristics – including virulence determinants, antimicrobial susceptibility, and epidemiological relevance – to regulatory trial sponsors and research ethics committees. Both strains have been extensively studied *in vitro* and *in vivo* and have served as references in numerous studies of *Salmonella* pathogenesis and host interactions, providing a strong evidence base to support their safety and suitability for use in a controlled human infection model (CHIM).

308 **3.2 GMP manufacture, quantification, and purity of challenge stocks**

309

310 Identification of manufactured challenge agent stocks was initially confirmed by positive
311 agglutination of both strains with *Salmonella* group B antiserum. Pre- and post-freeze viability
312 was confirmed by colony counting following serial dilutions. The pre-freeze viability of 4/74 was
313 1.24 x 10^9 CFU/ml, and the post-freeze viability was 5.2 x 10^8 CFU/ml. The pre-freeze viability of
314 D23580 was 9.95 x 10^8 CFU/ml, and the post-freeze viability was 4.9 x 10^8 CFU/ml.

315

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

321

322 **3.3 *S.* Typhimurium viability is reduced in acidic pH <3.5**

323

324 We next evaluated the optimal conditions for challenge agent viability and delivery, focusing on
325 the role of gastric acid pH as a first line of defence against enteric pathogens. Previous *S*. Typhi
326 challenge studies demonstrated that neutralising gastric acid prior to administration
327 significantly lowers the infectious ID_{50} dose [24]. Pre-treatment with sodium bicarbonate –
328 routinely used in human challenge models for *S*. Typhi [24], *Vibrio cholerae* [30], and *Shigella* spp.
329 [31] is also thought to reduce batch-to-batch variability and enhance reproducibility in enteric
330 challenge studies.

331

332 We began by assessing the pH sensitivity of D23580 and 4/74, with the aim of defining a threshold
333 below which viability of the challenge strains significantly decreases. 5.15x10^5 CFU/ml of each
334 strain were incubated for 15 minutes in several pH test conditions prior to plating. Both strains

335 demonstrated similar stability in 0.9% saline, sodium bicarbonate solution (2.6g/120ml), and
336 acidic buffer pH 5. Survival declined following incubation in pH ≤ 3.5, with both 4/74 and D23580
337 demonstrating a trend towards lower survival compared to other conditions **(Figure 1 – Panels**
338 **A-B).** These findings are consistent with known sensitivity of *Salmonella* to acidic conditions and
339 supports the commonly utilised approach of neutralising gastric acid with bicarbonate pre-
340 treatment in enteric challenge models [32,33].
341

342 **3.4 *S.* Typhimurium viability remains stable in 0.9% saline and bicarbonate buffer to 1**
343 **hour**
344

345 We sought to determine the stability of challenge strains under varying holding buffer conditions.
346 We began by comparing bacterial survival following incubation in 0.9% saline and sodium
347 bicarbonate (2.6g/120ml) buffer, in addition to testing different holding times on ice (T = 0, 1, 2, 3
348 hours) to determine the optimal solution and maximum hold duration to ensure viability at the
349 time of oral administration. Testing was initially performed with D23580, and the relevant optimal
350 holding conditions (0.9% saline; 1-hour hold) were repeated and confirmed for 4/74. Survival was
351 maintained at >90% in 0.9% saline solution to T=2hrs, dropping to <80% after T=3hrs, compared
352 with T=0hr baseline. There was a trend towards reduced survival in sodium bicarbonate
353 compared with 0.9% saline, with a marked drop in survival to <80% at T=2hrs **(Figure 1 – Panels**
354 **C-D)**. Similar survival >90% at T=1hr was demonstrated for strain 4/74. As a result of these
355 findings, we amended the challenge agent preparation SOP to use 0.9% saline solution instead
356 of sodium bicarbonate as the holding buffer with a maximum one-hour hold interval between
357 formulation and administration.

358 **3.5 Sodium bicarbonate pre-treatment maintains simulated gastric fluid pH above**
359 **critical thresholds for STm survival**
360

361 In the CHANTS study, participants are administered a challenge agent in the form of a liquid
362 suspension after fasting for at least 90 minutes. The challenge procedure comprises two
363 components: (i) a sodium bicarbonate solution (2.6g/120ml) pre-treatment for gastric acid
364 neutralisation, followed by (ii) a 30ml 0.9% saline solution-bacterial challenge agent suspension.
365

366 We next evaluated the impact of sodium bicarbonate pre-treatment on the expected pH of the
367 stomach at the time of bacterial challenge. Gastric acid represents a key defence mechanism
368 against gastrointestinal infection. *Salmonella enterica* have evolved multiple strategies to
369 improve survival following acid exposure, including the acid-tolerant response (ATR) and the
370 arginine D-carboxylase system, but the organism still remains susceptible to gastric acid killing
371 [32–34].
372

373 To model the gastric passage of the liquid challenge agent, we used the Rosset-Rice (RR) *in vitro*
374 system to model bacterial stability and survival following simulated transit through the stomach
375 and intestinal tract. The RR model is designed to replicate the low pH conditions of a fasted adult
376 stomach by introducing acid at rates that mimic physiological acid secretion. The model is
377 predicated on a set of assumptions concerning gastric physiology. In particular, the rate of
378 gastric emptying is known to be regulated by several factors (25). In fasted adults, it is estimated
379 that 240ml volume of non-caloric liquid can empty from the stomach in approximately 15-20
380 minutes [35,36]. The mean residual volume in a stomach after an overnight fast is estimated to
381 range between 25-50ml [35,37,38]. Allowing for inter-individual variability, the resting pH of a
382 fasted adult stomach typically ranges from pH 1 to 3 [39]. The basal acid output in healthy adult
383 stomachs is estimated to be 1-5 mmol HCl/hour, which increases significantly on eating [40].
384 Although it mimics some key aspects of gastric physiology, one limitation is that it does not

385 simulate the removal of gastric contents which would usually dilute the acidic environment as
386 contents are removed over time.
387
388 Gastric pH dynamics were modelled in the modified Rossett-Rice *in vitro* system modelling an
389 antacid dose of 2.6g sodium bicarbonate buffer to ensure that it maintains pH above the critical
390 threshold of pH 3.5 identified for D23580 and 4/74 survival. Serial measurements every minute
391 confirmed that gastric pH is maintained above the critical threshold pH >3.5 and is approximately
392 pH 6.5 at 70 minutes following administration of bicarbonate buffer **(Figure 2 – Panel A)**.
393

394 **3.6 *S.* Typhimurium viability remains stable in simulated gastric and intestinal fluids**
395 **after 1 hour hold on ice**
396

397 The modified Rossett-Rice model was then utilised to determine bacterial stability and survival
398 following simulated transit through the gastrointestinal tract. Challenge strains were incubated
399 in 0.9% saline solution or 2.6g/120ml sodium bicarbonate and either directly added to the RR
400 model or after a 1- or 2-hour hold on ice. Fluid was then sampled at 0, 30, 45 and 60 minutes after
401 addition to the RR model and plated for overnight incubation. Initial testing with D23580
402 demonstrated significant reduction in survival following T=2 hour on ice compared with T=0 hours
403 across all sampled time points ($p<0.05$) (**Supplementary Figure 1**). Subsequent testing
404 evaluated survival of both strains following T=0hr or T=1hr hold in ice in 0.9% saline. There were
405 no significant differences in survival between T=0hr and T=1hr hold on ice prior to addition to the
406 RR model. CFU counts declined with time and overall survival was approximately 70% and 80%
407 after 60 mins in the RR model for 4/74 and D23580, respectively (**Figure 2 – Panels B-C**).
408

409 We further modelled survival of the challenge strains during transit from the stomach to the small
410 intestine. STm challenge strains were again subjected to the RR model and after 70 minutes
411 samples were taken and exposed to simulated intestinal fluid and subsequently plated at 0, 30,

412 70-minute time points to mimic residence time in the upper small intestine. There was no
413 difference in survival of either challenge strain between 0-70 minutes of exposure to simulated
414 intestinal fluid (**Supplementary Figure 2**).
415

416 ## 3.7 Phenotypic Confirmation and Characterisation
417

418 ### 3.7.1 STm D23580 and 4/74 exhibit differential growth characteristics in melibiose
419

420 A key metabolic property distinguishing D23580 from 4/74 is the differential ability to utilise
421 disaccharide melibiose as a sole carbon source [28]. D23580 contains three nonsynonymous
422 SNPs in melibiose utilisation genes (*melB* and *melR*) leading to downregulated expression of
423 proteins responsible for active transport of melibiose across the cell membrane. It is speculated
424 that the ability of 4/74 to metabolise melibiose confers a survival advantage during
425 gastrointestinal colonisation, and the loss of this capacity in D23580 reflects niche adaptation
426 towards an invasive phenotype [28]. This differential utilisation was demonstrated by evaluation
427 of bacterial growth curves in minimal media and LB. Growth curves were comparable between
428 strains in minimal media supplemented with 5% glucose, whilst growth of STm D23580 is
429 markedly attenuated in minimal media supplemented with melibiose (**Error! Reference source**
430 **not found.** – **Panel A**).
431

432 ### 3.7.2 STm D23580 and 4/74 exhibit differential swimming motility
433

434 As previously described, swimming motility in soft agar is reduced in D23580 compared with 4/74
435 due to downregulation of flagellin expression [28]. This phenotype is consistent with other
436 invasive *Salmonella* serovars including Typhi, Paratyphi A, and Gallinarum, which rapidly reduce
437 flagellin expression upon cellular invasion to avoid immune recognition by TLR-5 and flagellin-
438 specific $CD4^+$ T-cells [41–43]. The reduced swimming motility of D23580 (halo diameter 2.7cm)

compared with 4/74 (4.5cm) in soft agar after 4 hours incubation at 37°C is illustrated in **Error! Reference source not found. – Panel B**.

3.7.3 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 behaviour that enhances *Salmonella* environmental stress resistance and biofilm formation. This loss of multicellular behaviour has been demonstrated in D23580, hypothesised to occur as a consequence of human host-adaptation [29]. A positive RDAR phenotype appears as a red colony with filamentous texture, whilst a negative RDAR phenotype appears as a smooth and yellow colony. Differential RDAR morphotype appearances between 4/74 and D23580 are illustrated in Error! Reference source not found. **– Panel C**.

3.7.4 Antibiotic Susceptibility

Multi-drug resistance in *Salmonella spp.* is defined as resistance to antibiotics from three or more separate classes [44]. iNTS is increasingly multi-drug resistant in Africa, demonstrated by the emergence and persistence of ST313 [5]. D23580 harbours four plasmids, including pSLT-BT, which encodes resistance to chloramphenicol, ampicillin, streptomycin, sulphonamides, and trimethoprim.

Disc diffusion on Mueller-Hinton agar, and broth microdilution using BD Phoenix provided zone inhibition diameters and MIC concentrations respectively and were compared to published EUCAST guidelines for sensitivity [27]. Antibiotic sensitivity results for both strains were as predicted in both disc diffusion and broth microdilution methods. BD Phoenix minimum inhibitory concentrations (MIC) and interpretations are illustrated in **Table 2**.

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

469 **3.8 Whole genome sequencing confirms STm genomic stability post-manufacture**
470

471 Illumina whole-genome sequencing was performed to assess for single nucleotide
472 polymorphism (SNP) differences which may have arisen during the manufacturing process
473 compared with established reference strain sequences.
474

475 No SNP mutations were detected comparing *S*. Typhimurium 4/74 or D23580 manufactured
476 stocks with the reference strain sequences [4/74 Genbank Accession Numbers: CP002487.1 -
477 CP002490.1; D23580 Genbank Accession Numbers: LS997973.1 – LS997977.1], highlighting
478 overall genomic stability. Consistency with genetic sequences from reference strains ensures
479 the manufactured stocks administered to participants encode the same repertoire of virulence
480 factors as circulating strains causing iNTS in sSA. Moreover, these strains encode the putative
481 key antigens targeted by vaccine candidates in development, including the *wbaV, wbaU, wbaN,*
482 *wzxB1* genes in the O-antigen biosynthesis cluster encoding O4, alongside *fliC* and *fliB* genes
483 encoding phase variable flagellar antigens.
484

485 **3.9 Sodium bicarbonate exposure does not impact key STm virulence gene expression**
486

487 Administration of sodium bicarbonate pre-treatment is frequently utilised in enteric pathogen
488 CHIMs to lower the required challenge inoculum, and to reduce batch-to-batch variation
489 [24,45,46]. We have demonstrated that STm challenge strain survival is stable, and gastric pH is
490 maintained above critical thresholds in a simulated gastrointestinal tract. However, sodium
491 bicarbonate exposure has also been shown to have a significant effect on bacterial virulence
492 factor gene expression in other enteric pathogens, notably in *E. coli* spp, *Citrobacter,* and *V.*
493 *cholerae* [47–50]. We investigated the impact of sodium bicarbonate exposure on *S.*
494 Typhimurium virulence gene expression; in case it altered the virulence of challenge agent stocks
495 at the time of oral administration.

496 Transcriptomic profiling of STm D23580 and 4/74 has previously been undertaken and
497 characterised in multiple infection-relevant conditions [28]. In this investigation, we performed
498 RNA-seq on whole RNA extracted from STm D23580 and 4/74 after 30-minutes incubation in
499 sodium bicarbonate solution (0.53g/30ml) and compared gene expression levels to cultures
500 which had not been exposed to bicarbonate. These conditions were selected for testing to reflect
501 the anticipated duration bacterial cells would be in contact with bicarbonate buffer during transit
502 from the stomach to the small intestine following oral challenge.
503
504 The transcriptional profiles of both strains were evaluated and individual gene expression level
505 calculated as transcripts per million (TPM). A gene is considered as expressed when TPM is
506 greater than 10 [28]. The proportion of expressed genes decreased after incubation in sodium
507 bicarbonate solution for both 4/74 and D23580 (4.11% for 4/74 and 8.15% for D23580). Both
508 strains expressed approximately 70% genes (TPM >10) after 30 minutes incubation in sodium
509 bicarbonate solution compared with water (**Supplementary Figure 3**).
510
511 *Salmonella* pathogenicity islands SPI-1 and SPI-2 play major roles in human infection [51,52],
512 with SPI-1 being required for bacterial invasion of epithelial cells and SPI-2 mediating the
513 formation and maintenance of *Salmonella*-containing vacuoles (SCVs) [53]. Flagella are also
514 major virulence factors during *Salmonella* infection [54]. It has been shown that sodium
515 bicarbonate exposure down-regulates flagella expression in *E. coli* [50]. To determine the impact
516 of sodium bicarbonate exposure on *Salmonella* virulence gene expression, we used a differential
517 expression approach to compare gene expression before and after sodium bicarbonate
518 treatment and determined that 6% of 4/74 genes and 5% of D23580 genes were differentially
519 expressed. No significant up-regulation or down-regulation of SPI-1, SPI-2 or flagellar genes was
520 detected in either strain following bicarbonate exposure. To determine any likely functional
521 impact of the bicarbonate-regulated genes, we examined gene ontology and determined that

522 there were no common functional links among the genes (**Figure 4**). Our data suggest that the
523 minor changes in gene expression that were observed are more likely to reflect experimental
524 variability than a meaningful biological trend. In summary, the transcriptomic data suggest that
525 exposure to sodium bicarbonate does not significantly alter *Salmonella* virulence.
526
527

528 # 4 Discussion and Conclusions

529

530 Bacterial stocks used for controlled human infection studies need to be manufactured to a high
531 standard. In this study, we have described the processes involved with GMP manufacture and
532 comprehensive phenotypic, genomic and transcriptomic characterisation of two *S.* Typhimurium
533 strains - D23580 (ST313) and 4/74 (ST19). The steps described here are comparable with those
534 undertaken for previous *Salmonella* challenge studies and serve as a benchmark for the type of
535 testing required for new challenge agent development. Phenotypic and genotypic
536 characterisation demonstrated that these two strains behaved as expected and no significant
537 changes were introduced during the manufacturing process, giving us confidence prior to their
538 administration to healthy human volunteers. We identified that both strains demonstrated
539 improved survival in 0.9% saline holding buffer compared with sodium bicarbonate,
540 subsequently adapting our challenge protocols in response to these data, modifying approaches
541 used in previous *Salmonella* challenge models. A novel aspect of this study is a more detailed
542 characterisation of *Salmonella* challenge stocks under conditions mimicking those encountered
543 in the gastrointestinal tract using a modified Rossett-Rice model. The addition of a
544 transcriptomic assessment of virulence gene expression offers a snapshot of the expected
545 transcriptional state of the challenge agent as it enters the stomach, providing new insights and
546 assurances that key virulence factors are unchanged in *Salmonella* following sodium
547 bicarbonate pre-treatment which is a requirement of accepted enteric challenge model
548 protocols.

549

550 The strains used in this model were selected with careful consideration of the principles outlined
551 in **Table 1**. An ideal challenge model would incorporate multiple diverse strains to better reflect
552 pathogen diversity. Whilst this approach might be optimal, it is generally acknowledged that
553 practical limitations, including GMP manufacturing costs and regulatory complexity, often

554 preclude challenge studies from using more than one strain. Consequently, a detailed
555 characterisation of well-documented strains is considered the most feasible approach in most
556 circumstances. Provenance, traceable history from isolation, and prior comprehensive
557 characterisation provides greater assurance in the safety and reliability of this first-in-human
558 CHIM. *S*. Typhimurium was chosen as the preferred serovar for use in this model as it is
559 responsible for the greatest burden of iNTS in sSA and is supported by the most comprehensive
560 *in vitro* characterisation data.
561
562 Utilising two distinct strains of *S*. Typhimurium offers a unique opportunity to evaluate the
563 clinical, microbiological and immunological outcomes following challenge in a healthy immune
564 competent population. This platform facilitates direct comparison between strains and therefore
565 offers a deeper understanding of the possible impact of genome degradation and human-
566 adaptation observed in D23580, which may partly account for its propensity to cause invasive
567 disease in sSA [6]. This also supports our approach to using wild-type D23580 which harbours a
568 distinct plasmid and prophage repertoire compared with 4/74. Whilst this strain is classified as
569 multi-drug resistant, it remains fully sensitive to all clinically relevant antibiotics used in the
570 CHANTS study, ensuring participant safety.
571
572 In this study, challenge agent manufacture has been undertaken to GMP standard. At the time of
573 writing, the UK does not legally require challenge agents to be produced under GMP. Approval
574 from the UK Medicines and Healthcare products Regulatory Agency (MHRA) is only necessary if
575 the CHIM study involves an investigational medicinal product, such as if a vaccine is
576 administered alongside the challenge agent. Nevertheless, the MHRA guidance generally
577 recommends following GMP principles to ensure the safety and quality of the produced
578 challenge agent. Oversight primarily rests with the ethics committee and study sponsors [55,56].
579

580 *Salmonella* Typhimurium is often ascribed as one of the most comprehensively studied and
581 characterised pathogens and is a model organism for bacteriological research. Availability of
582 such a compendium of characteristic data was leveraged to facilitate the comprehensive
583 phenotypic and genomic characterisation undertaken in this study [57]. *In vitro* phenotypic
584 properties were confirmed to be consistent with published data including RDAR morphotype,
585 swimming motility and growth kinetics in a variety of nutrient media. Antimicrobial susceptibility
586 testing also confirmed activity of antibiotic agents selected for use within the CHANTS study.
587 Further, whole genome sequencing has confirmed genomic stability of both strains throughout
588 the manufacturing process and confirmed no genetic modifications which might alter expected
589 pathogenicity. Longitudinal sequencing of stored challenge agent stocks is also being
590 undertaken throughout the duration of the CHANTS study to confirm maintained genomic
591 stability.
592
593 Building on work from previous *Salmonella* challenge models, we have undertaken novel
594 assessments of the stability and viability of *Salmonella* in a range of pH conditions, holding
595 buffers, and in a simulated gastrointestinal environment, to model survival and phenotype at the
596 point of oral administration.
597
598 Our data indicated differences in the stability of *S*. Typhimurium in holding buffers compared with
599 previous *S*. Typhi and Paratyphi studies. Recent typhoidal *Salmonella* challenge studies
600 administer 2.1g/120ml of sodium bicarbonate buffer followed by the challenge agent in a
601 0.53g/30ml sodium bicarbonate holding buffer [45]. The data presented here indicate improved
602 bacterial survival in a 0.9% saline holding buffer compared with sodium bicarbonate. As a result,
603 the sample preparation SOP was updated so that the *S*. Typhimurium challenge agent is
604 administered in 30ml 0.9% saline, similar to the holding buffer used in recent *Shigella sonnei*
605 challenge studies [58]. In parallel, the bicarbonate buffer protocol was adjusted to account for

606 the reduction in sodium bicarbonate that would have been otherwise used as a holding buffer for
607 the 30ml *S*. Typhimurium inoculum. We now administer 2.6g/120ml sodium bicarbonate
608 solution, which provides the same total mass of bicarbonate ensuring equivalent buffering
609 capacity compared to previous *Salmonella* challenge study SOPs [45].
610
611 Bacterial cells remained stable in pH >3.5, stable for up to 2 hours in 0.9% saline holding buffer
612 and 1 hour in sodium bicarbonate and remained stable up to 60 minutes in a modified Rossett-
613 Rice model. These key findings provide assurance that viable bacterial cell counts remain stable
614 during the challenge agent preparation, transfer and administration protocols followed in the
615 CHANTS study. Furthermore, we have demonstrated that exposure to bicarbonate buffer does
616 not significantly alter *Salmonella* virulence factor gene expression. These findings taken together
617 provide assurance that bacterial cell concentration remains stable during challenge agent
618 preparation and transfer from the laboratory to clinic, and that gastric acid neutralisation with
619 bicarbonate pre-treatment does not impact pathogenicity at the point of oral administration,
620 thus ensuring close recapitulation of what likely occurs in natural exposure.
621
622 Whilst this study has provided a comprehensive characterisation of STm 4/74 and D23580 for
623 use in a first-in-human CHIM, there are limitations in our approach which should be considered.
624 Firstly, contemporary epidemiological genomic and surveillance studies highlight the important
625 contributions *S*. Enteritidis – and the emergence of *S*. Typhimurium ST313 sub lineages 2.2 and
626 2.3 – have in iNTS disease burden [5,59]. Including these strains in our model could further
627 enhance generalisability to those isolates currently circulating in sSA, which account for a
628 significant proportion of iNTS disease. Furthermore, using recently harvested isolates – or
629 attenuated strains – could potentially compromise the rigorous ethical standards adhered to in
630 the development of CHIMs, as highlighted in **Table 1**. As such, strain selection was based upon

631 these core principles, with STm 4/74 and D23580 being selected as priority pathogens which
632 could provide the greatest translational data within the context of a CHIM.
633
634 In developing a non-typhoidal *Salmonella* (NTS) challenge model to mimic invasive disease, we
635 opted to develop two strains of *S*. Typhimurium for administration to volunteers, as the serovar
636 responsible for the majority of iNTS cases globally. Future plans include the development of a
637 *Salmonella* Enteritidis challenge model, which accounts for approximately one third of iNTS
638 cases worldwide. Like *S*. Typhimurium, *S*. Enteritidis strains associated with invasive disease in
639 low-income settings, including *S*. Enteritidis ST11 [60], have distinct genetic profiles compared
640 with those causing gastrointestinal disease in high-income regions. The strain selection,
641 development and manufacture of such a strain would follow principles similar to those outlined
642 here.
643
644 In summary, extensive characterisation of both *S*. Typhimurium D23580 and 4/74 has
645 experimentally confirmed predicted and known properties of these strains. These investigations
646 have significantly advanced the development of *Salmonella* human infection models and
647 support ongoing efforts to gain deeper understanding of host-pathogen interactions and immune
648 correlates of protection. Gaining such new understanding of iNTS infection can strengthen
649 ongoing efforts to support iNTS vaccine and therapeutic development. We aim to report CHANTS
650 study primary outcome data in the near future and will outline how these findings could be
651 translated to support ongoing efforts to combat iNTS disease in sSA.
652

5 Declarations

Ethics Statement

This CHANTS study protocol has been reviewed and approved by the NHS Health Research Authority (London—Fulham Research Ethics Committee 21/PR/0051; IRAS 301659) and is registered on ClinicalTrials.gov (registration number NCT05870150). Study sponsor Imperial College London.

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Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Genbank Accession Numbers (Reference Genomes):

Salmonella Typhimurium 4/74 CP002487.1 - CP002490.1

Salmonella Typhimurium D23580 LS997973.1 – LS997977.1

Raw RNA-seq data from bicarbonate experiments have been submitted to the NCBI Gene Expression Omnibus (GEO) Repository. Accession codes are currently being generated, are available from the corresponding authors, and will be included in a final published manuscript.

Competing Interests

The authors have declared that no competing interests exist.

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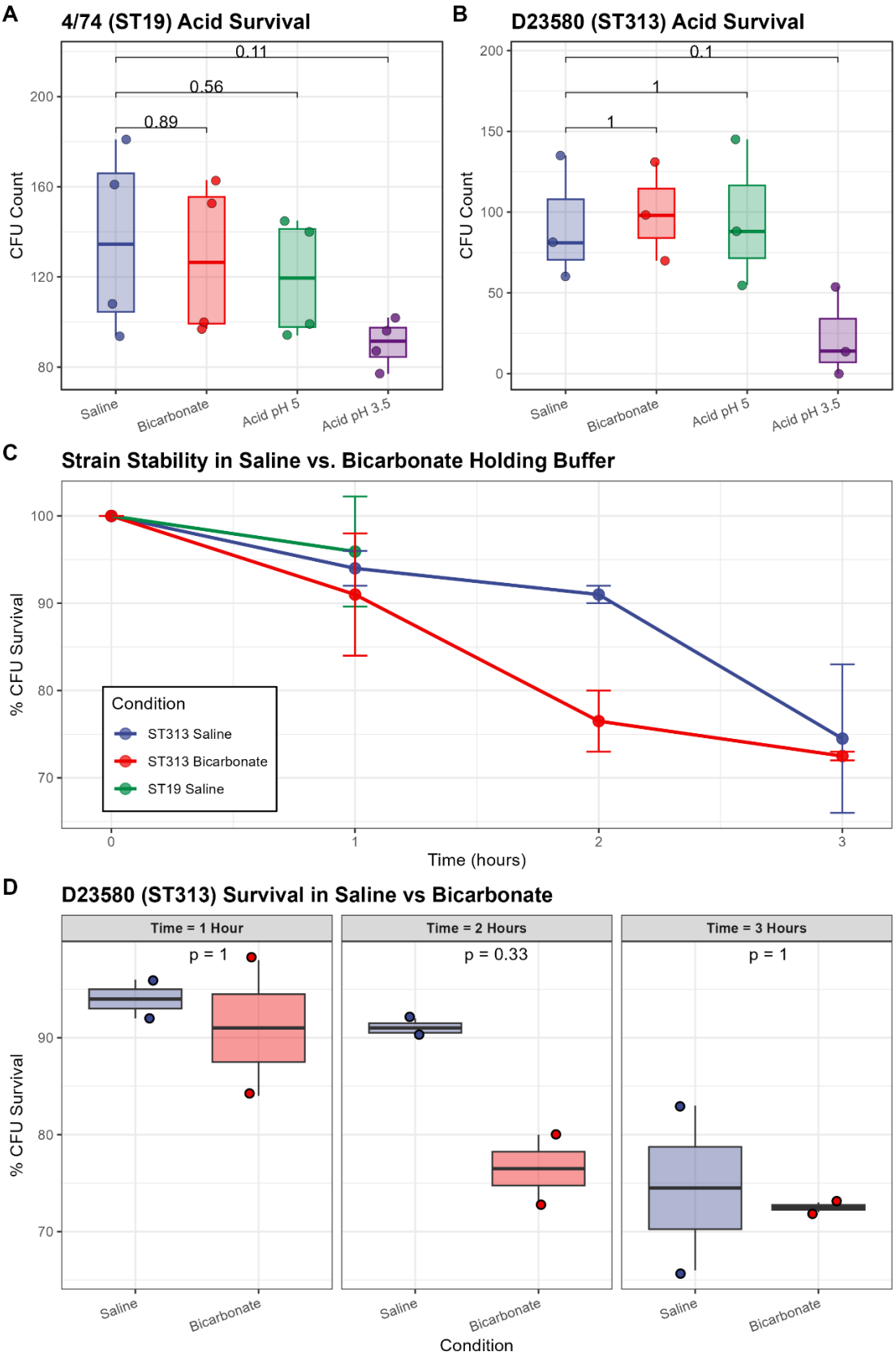
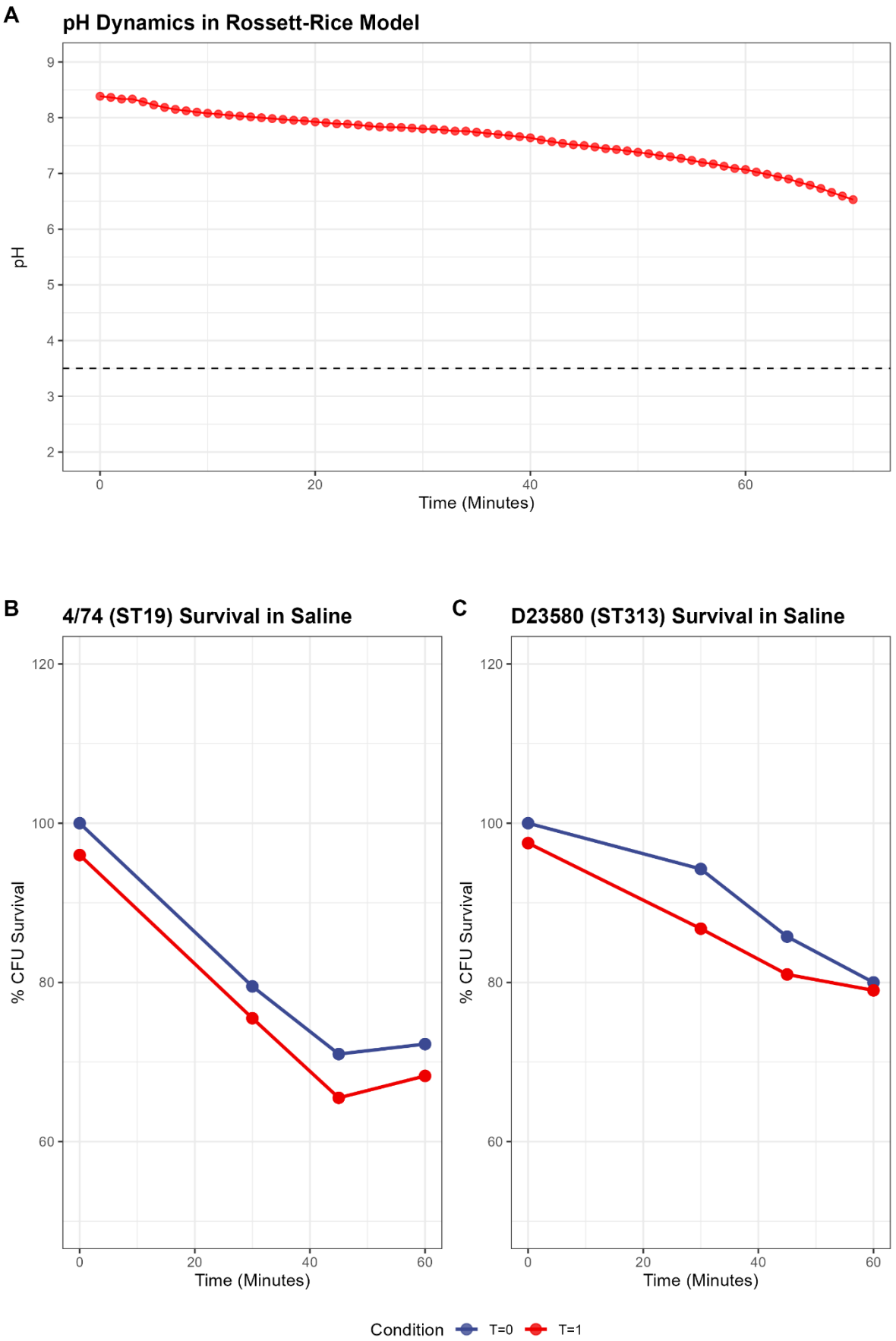
7 Figures


A
4/74 (ST19) Acid Survival
0.11
0.56
0.89
CFU Count
200
160
120
80
Saline
Bicarbonate
Acid pH 5
Acid pH 3.5
B
D23580 (ST313) Acid Survival
0.1
1
1
CFU Count
200
150
100
50
0
Saline
Bicarbonate
Acid pH 5
Acid pH 3.5
C
Strain Stability in Saline vs. Bicarbonate Holding Buffer
% CFU Survival
100
90
80
70
Condition
ST313 Saline
ST313 Bicarbonate
ST19 Saline
0
1
2
3
Time (hours)
D
D23580 (ST313) Survival in Saline vs Bicarbonate
Time = 1 Hour
Time = 2 Hours
Time = 3 Hours
p = 1
p = 0.33
p = 1
% CFU Survival
90
80
70
Saline
Bicarbonate
Condition

908 **Figure 1 – Acid pH sensitivity and stability of 4/74 and D23580 in saline vs. sodium bicarbonate holding**
909 **buffers.** (A) *S.* Typhimurium 4/74 and (B) D23580 survival following incubation for 15 minutes in 0.9%
910 saline, 2.6g/120ml sodium bicarbonate, and acidic buffer pH 5 and pH 3.5. 0.9% saline served as the
911 control buffer for pairwise comparisons. Boxplots represent median and interquartile range. (C) D23580
912 and 4/74 survival in 0.9% saline compared with 2.6g/120ml sodium bicarbonate buffer over time.
913 Percentage CFU survival compared as a proportion with T=0hrs. D23580 was tested across all time points
914 and buffer conditions. 4/74 was tested only in saline at T=0 and T=1hr following selection of the optimal
915 conditions. (D) D23580 survival contrasting 0.9% saline with bicarbonate across timepoints. Boxplots
916 represent median and interquartile range. P = two-sided Mann-Whitney U test.


A
pH Dynamics in Rossett-Rice Model
pH
Time (Minutes)
B
4/74 (ST19) Survival in Saline
% CFU Survival
Time (Minutes)
C
D23580 (ST313) Survival in Saline
% CFU Survival
Time (Minutes)
Condition
T=0
T=1

917

918 **Figure 2 – Simulated gastric fluid pH and D23580 and 4/74 survival in the modified Rossett-Rice *in***
919 ***vitro* system**. (A) Simulated gastric fluid pH dynamics in the RR model following administration of 0.26g
920 bicarbonate. A peristaltic pump (set at a rate of 0.4 ml per minute) was used to add additional SGF
921 throughout the experiment to mimic conditions in the stomach, with pH monitoring every minute. pH >6.5
922 is maintained to 70 minutes. (B) 4/74 and (C) D23580 survival in the modified Rossett-Rice in vitro system,
923 simulating bacterial transit through the upper gastrointestinal tract following T=0/T=1hr pre-incubation in
924 saline. Mean % CFU survival calculated from 4 technical replicates per time point.

925

926

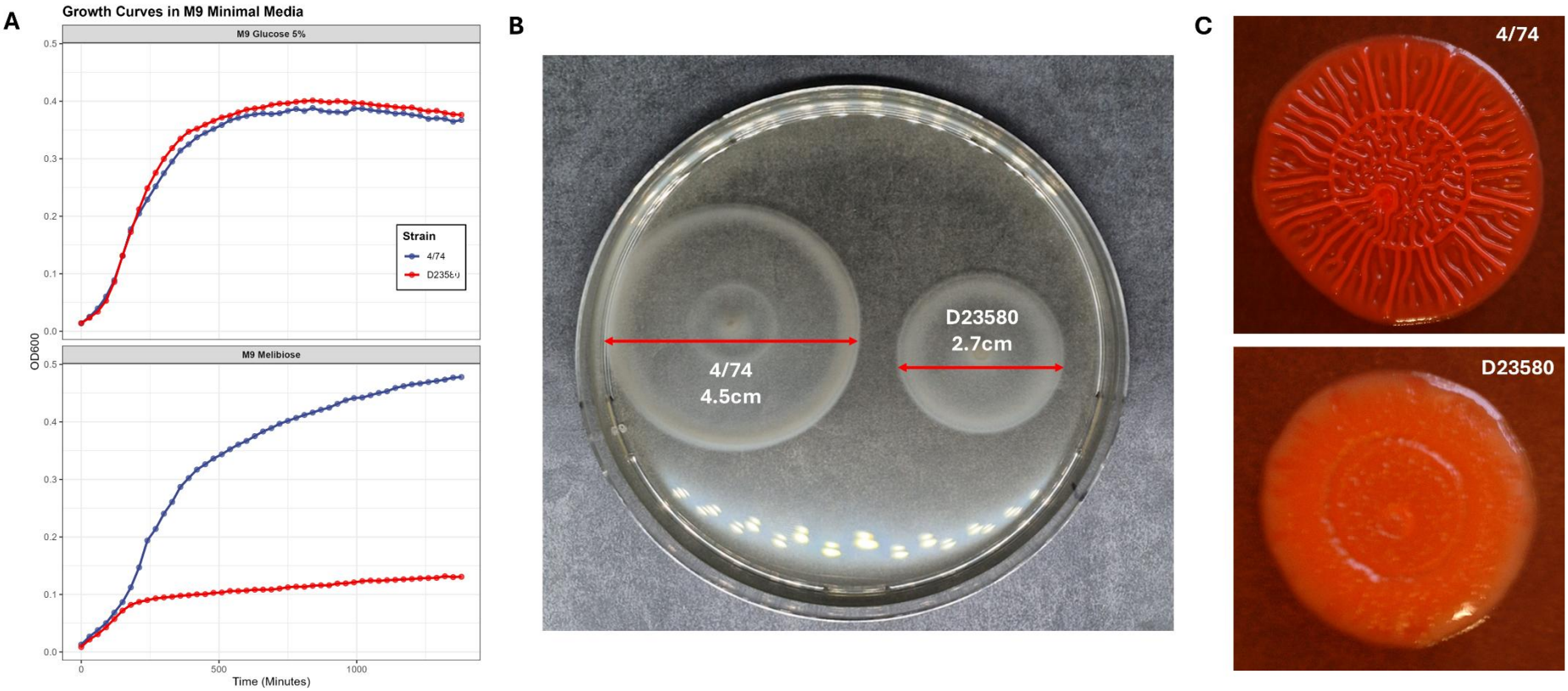
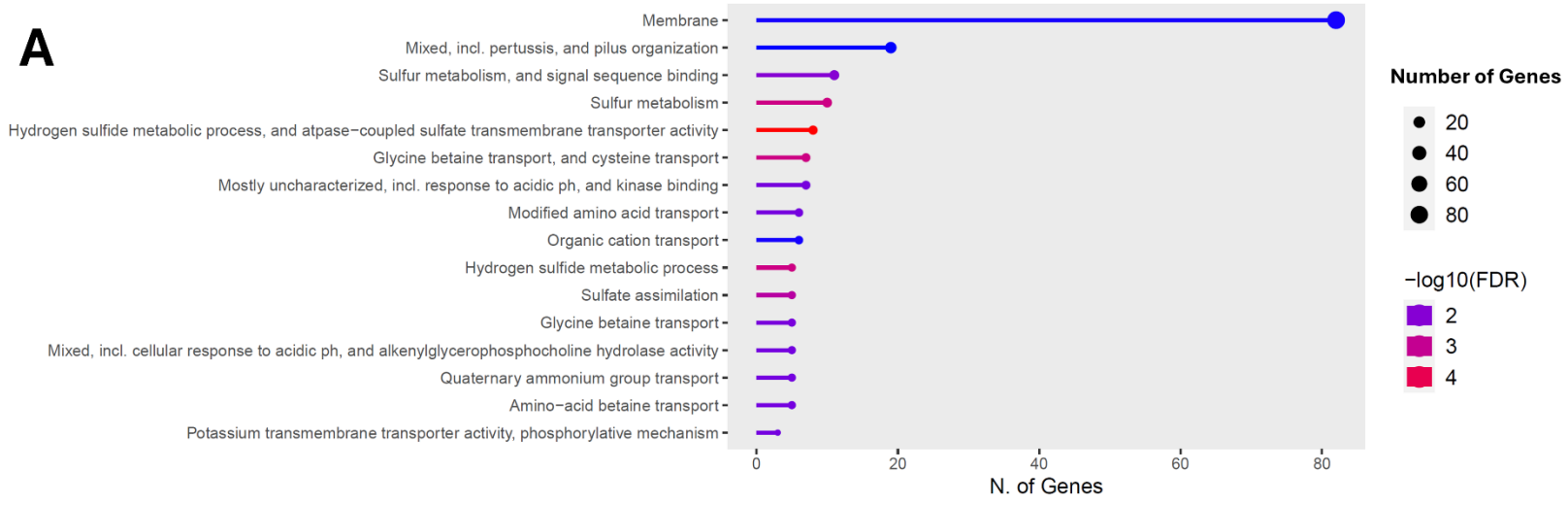
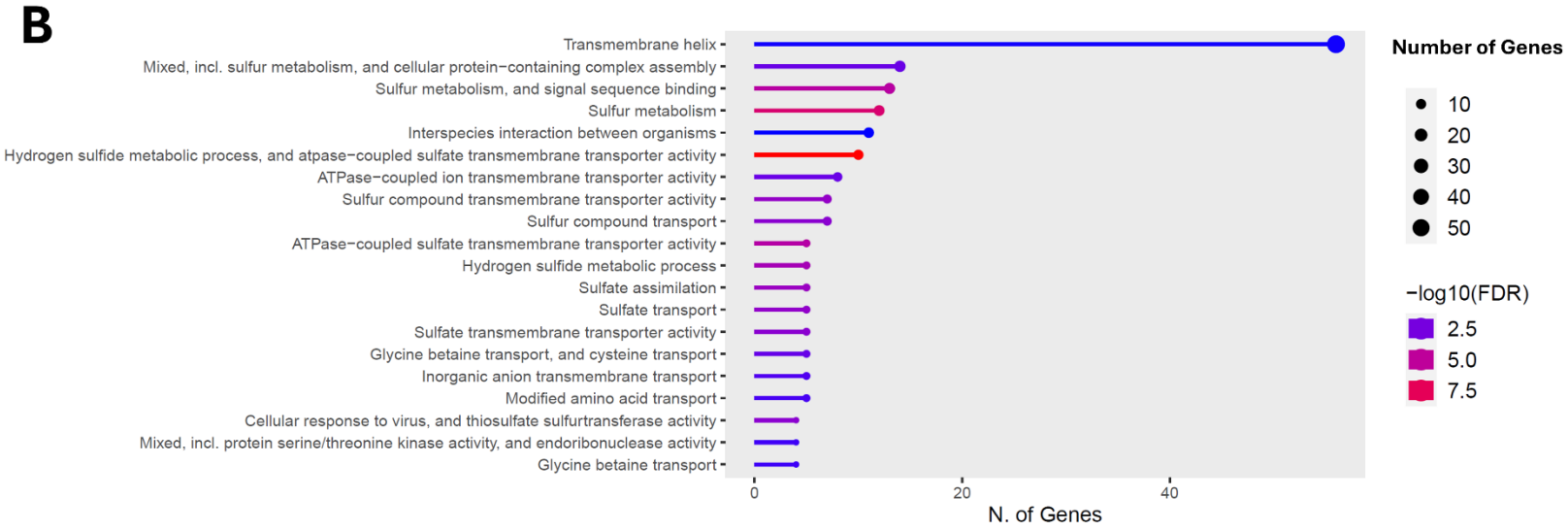


Figure 3 – Phenotypic characterisation of STm 4/74 and D23580. (A) Growth curves for 4/74 and D23580 in M9 minimal media supplemented with 5% glucose (top) and melibiose (bottom). Analysed by Enzyscreen Growth Profiler over 24 hours, starting OD600 = 0.01. Comparable growth patterns are observed in 5% glucose, whilst growth of D23580 is inhibited in melibiose. Mean OD600 values at each time point calculated from six technical replicates. (B) Swimming motility of 4/74 (left) and D23580 (right) in 0.3% LB agar. Photographed after 4 hours incubation at 37°C. (C) Red, dry and rough (RDAR) morphotype colonies of (B) 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.

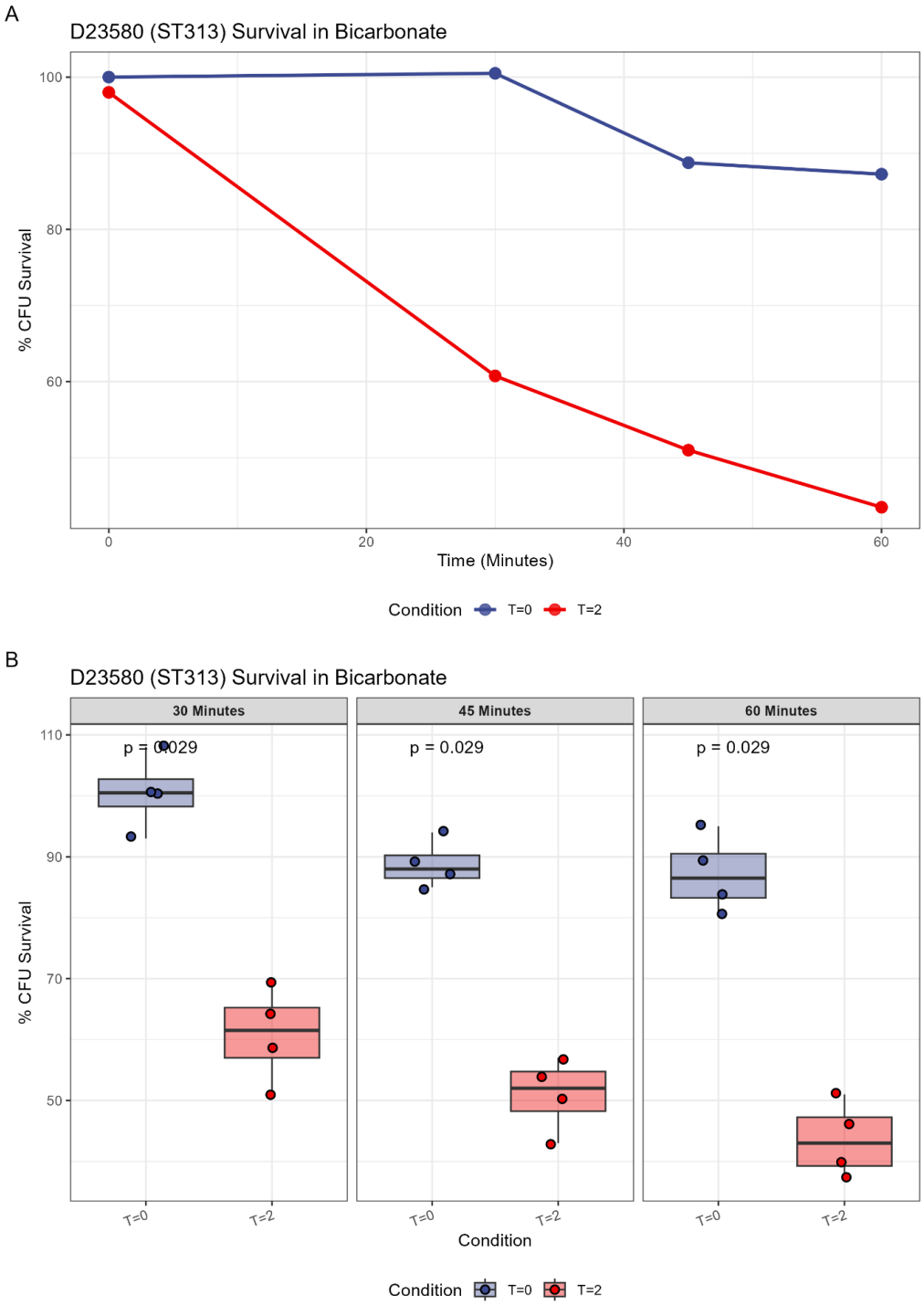

A
Membrane
Mixed, incl. pertussis, and pilus organization
Sulfur metabolism, and signal sequence binding
Sulfur metabolism
Hydrogen sulfide metabolic process, and atpase−coupled sulfate transmembrane transporter activity
Glycine betaine transport, and cysteine transport
Mostly uncharacterized, incl. response to acidic ph, and kinase binding
Modified amino acid transport
Organic cation transport
Hydrogen sulfide metabolic process
Sulfate assimilation
Glycine betaine transport
Mixed, incl. cellular response to acidic ph, and alkenylglycerophosphocholine hydrolase activity
Quaternary ammonium group transport
Amino−acid betaine transport
Potassium transmembrane transporter activity, phosphorylative mechanism
0
20
40
60
80
N. of Genes
Number of Genes
20
40
60
80
−log10(FDR)
2
3
4


B
Transmembrane helix
Mixed, incl. sulfur metabolism, and cellular protein−containing complex assembly
Sulfur metabolism, and signal sequence binding
Sulfur metabolism
Interspecies interaction between organisms
Hydrogen sulfide metabolic process, and atpase−coupled sulfate transmembrane transporter activity
ATPase−coupled ion transmembrane transporter activity
Sulfur compound transmembrane transporter activity
Sulfur compound transport
ATPase−coupled sulfate transmembrane transporter activity
Hydrogen sulfide metabolic process
Sulfate assimilation
Sulfate transport
Sulfate transmembrane transporter activity
Glycine betaine transport, and cysteine transport
Inorganic anion transmembrane transport
Modified amino acid transport
Cellular response to virus, and thiosulfate sulfurtransferase activity
Mixed, incl. protein serine/threonine kinase activity, and endoribonuclease activity
Glycine betaine transport
0
20
40
N. of Genes
Number of Genes
10
20
30
40
50
−log10(FDR)
2.5
5.0
7.5

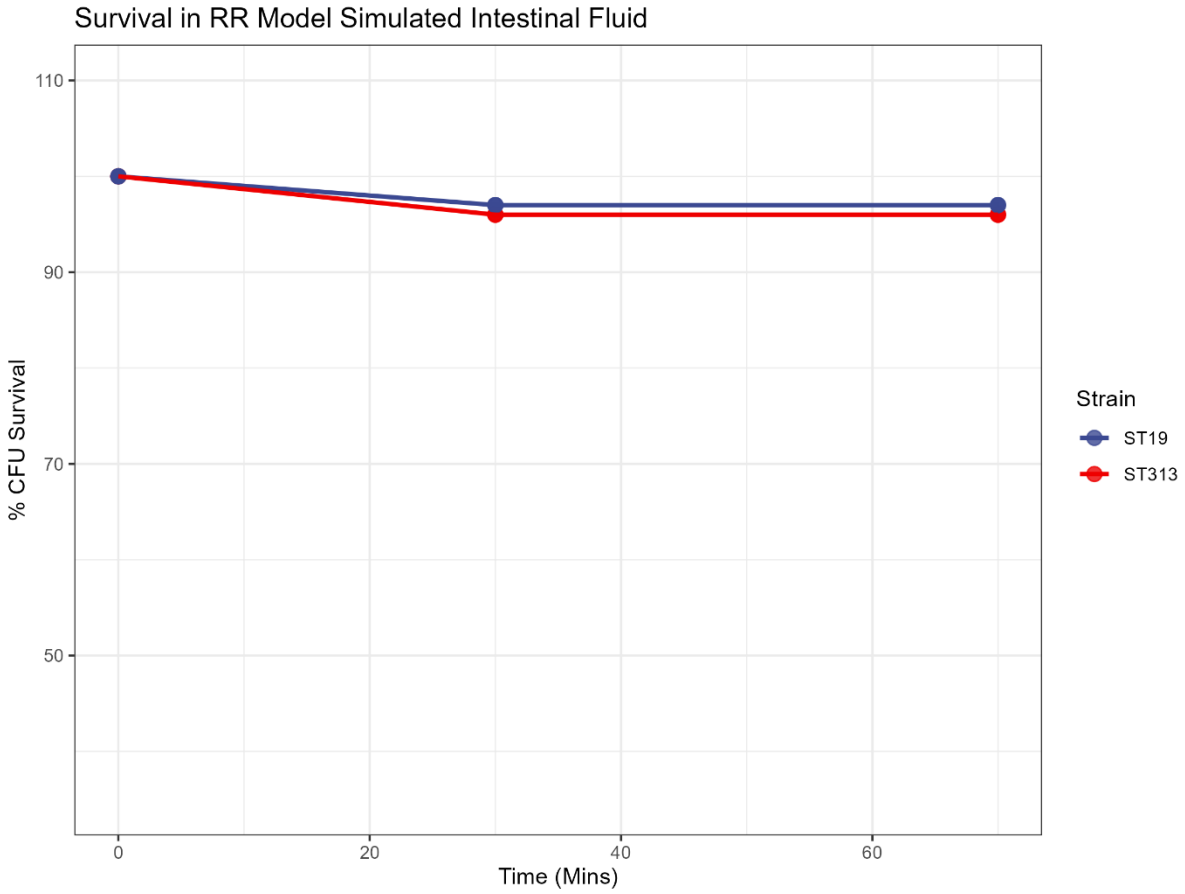
933

934 **Figure 4** – (A) STm 4/74 (ST19) and (B) D23580 (ST313) functional ontology of genes that were significantly differentially expressed following exposure to bicarbonate.
935 Membrane and transmembrane helix associated genes were affected with greatest frequency; no differentially expressed genes were associated with SPI-1, SPI-2
936 or flagellar virulence genes.

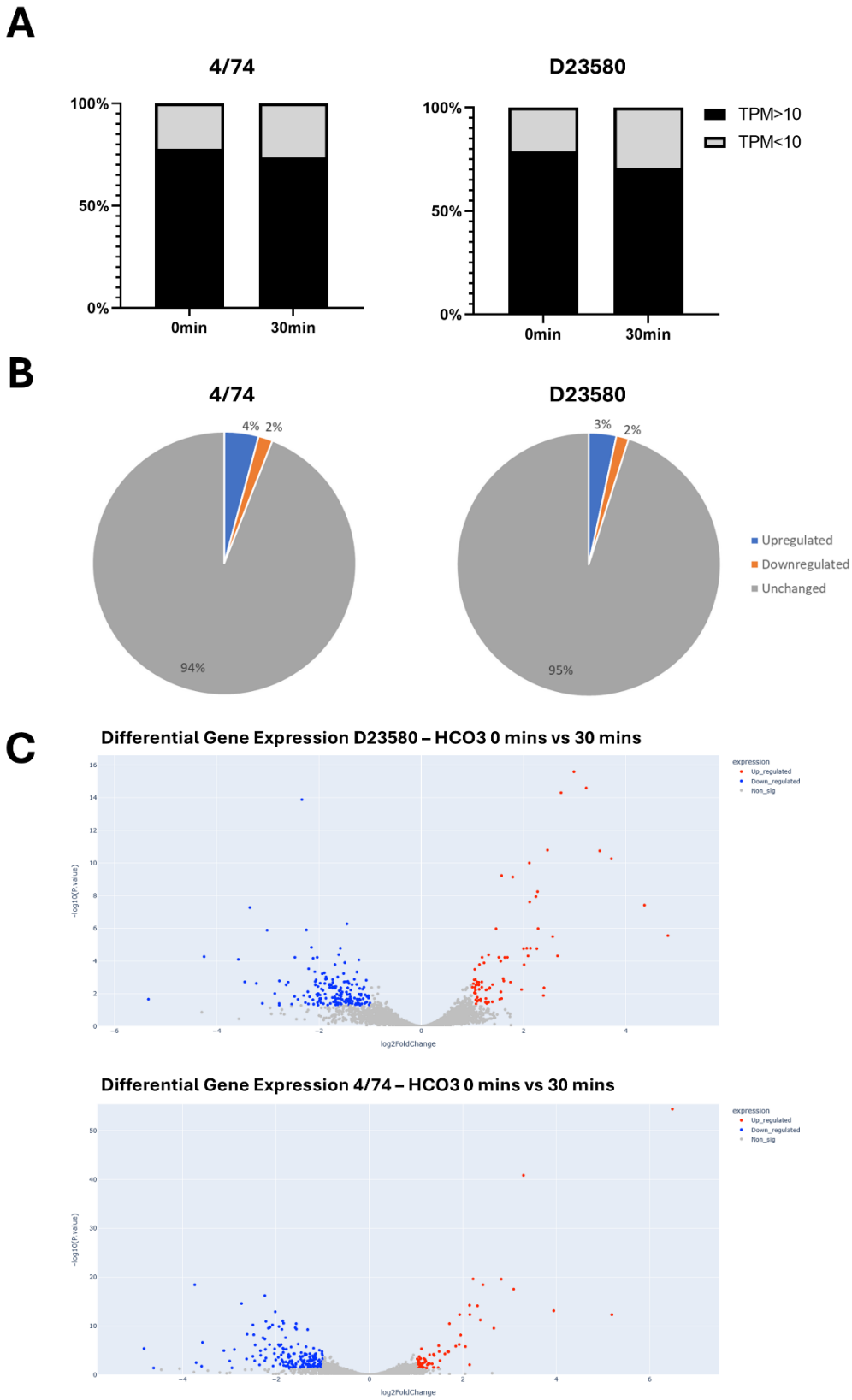
8 Supplementary Figures



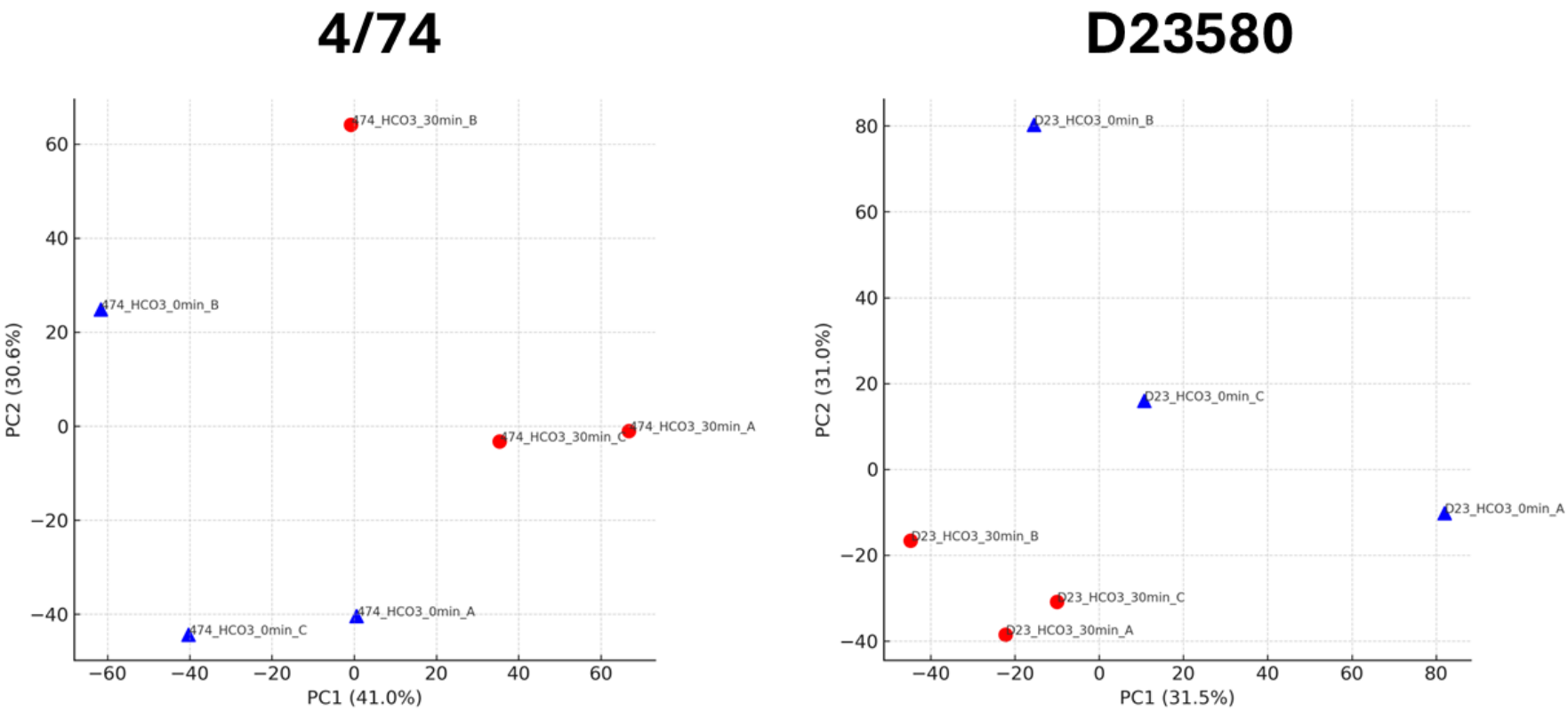
Supplementary Figure 1: (A) and (B) represent D23580 survival over time following incubation in 2.6g/120ml sodium bicarbonate solution for either T=0hr or T=2hr prior to addition to the RR model. Boxplots represent median and interquartile range. P = two-sided Mann-Whitney U test.



Supplementary Figure 2: Percentage CFU survival of 4/74 (ST19) and D23580 (ST313) following incubation in simulated intestinal fluid to model passage through the upper gastrointestinal tract.



Supplementary Figure 3: (A) Relative gene expression levels of 4/74 (left) and D23580 (right) in TPM. (B) Proportion of significantly up- or down-regulated genes in 4/74 (left) and D23580 (right) after 30-minutes exposure to sodium bicarbonate. (C) Volcano plots representing differentially expressed genes. Interactive plots with gene label names are available at https://github.com/Adalijuanluo/CHANTS_RNAseq.



Supplementary Figure 4: Principal component analyses representing variance in gene expression patterns in 4/74 (left) and D23580 (right) after 0-minutes or 30-minutes incubation in sodium bicarbonate solution.

957