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# Comparison of global transcriptomes for nontyphoidal *Salmonella* clinical isolates from pediatric patients with and without bacteremia after their interaction with human intestinal epithelial cells *in vitro*

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ABSTRACT

*Background:* Nontyphoidal *Salmonella* (NTS) outbreaks of invasive diseases are increasing. Whether the genetic diversity of invasive NTS correlates with the clinical characteristics and bacteremia development in NTS infections remains unclear. In this study, we compared the global transcriptomes between bacteremic and non-bacteremic NTS strains after their interaction with human intestinal epithelial cells *in vitro*.

*Methods:* We selected clinical isolates obtained from stool and blood samples of patients with or without bacteremia and patients with high and low C-reactive protein (CRP) levels. The bacterial RNA samples were isolated after coculturing with Caco-2 cells for RNA sequencing and subsequent analyses.

*Results:* CRP is an unreliable predictive maker for NTS bacteremia with a median CRP level of 1.6 mg/dL. Certain *Salmonella* Pathogenicity Island (SPI)-1 genes (*sipC*, *sipA*, *sicA*, *sipD*, and *sipB*), SPI-2 genes (*ssaP*, *ssrA*, and *ssaS*), and six SPI-4 genes (*siiA*, *siiB*, *siiC*, *siiD*, *siiE*, and *siiF*) remained upregulated in the bacteremic blood-derived strains but significantly downregulated in the nonbacteremic strains after their interaction with Caco-2 cells. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analysis identified that arginine biosynthesis, ascorbate and aldarate metabolism, and phosphotransferase system pathways were activated in bacteremic NTS strains after Caco-2 cell priming.

*Conclusion:* CRP levels were not correlated with bacteremia development. Significant regulation of certain SPI genes in bacteremic NTS strains after Caco-2 cell priming; bacteremia development might be influenced by the host immune response and the extent to which specific metabolism pathways in NTS strains can be prevented from invading the bloodstream.

## 1. Introduction

Nontyphoidal *Salmonella* (NTS) is a common pathogen in humans and animals worldwide and is associated with high rates of morbidity and mortality.[1] NTS bacteremia is a type of invasive NTS (iNTS) infection, with different prevalence rates in different countries. The highest prevalence (19 %) of iNTS was detected among individuals with community-acquired bloodstream infections in Africa, with NTS being more common than typhoidal *Salmonella* and being responsible for nearly 60 % of *Salmonella* bloodstream infections.[2] Prognostic factors for NTS bacteremia in adults include systemic lupus erythematosus; Group B, C, or D NTS infection; immunodeficiency[3]; high rates of resistance to

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readily available antibiotics; coma; and inadequate antibiotic treatment.[4] However, these reports have merely provided analytical summaries for predicting NTS bacteremia.

High-throughput technologies are increasingly being used to investigate iNTS infection. In a study, DNA microarrays of the bacteremic strains of *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S*. Typhimurium) demonstrated a common core of virulence genes, which might be required for invasive salmonellosis.[5] Another study used whole-genome sequencing to examine *Salmonella* invasiveness in *S*. Dublin, and the results revealed several virulence factors, but no genomic markers were detected for differentiating between invasive and noninvasive isolates.[6] This finding suggests that in addition to the genes themselves, the host immune response and the expression of NTS virulence genes after host immunity is primed affect the clinical outcome of iNTS infection. The infection process of NTS occurs in various hostile environments within the host, including the acidic stomach, and in the presence of intestinal bile acids, antimicrobial peptides, and toxic intracellular vacuoles.[7] NTS employs extensive host-adaptive responses for increased virulence and survival in the host, including fimbriae, toxins, pathogenicity islands, and virulence-associated plasmids. Although genomic analyses have advanced our understanding of the genetic diversity of iNTS, the cause of the clinical differences in NTS infections, including bacteremia development, remains unclear.[8]

A few studies have used RNA sequencing (RNA-seq) to investigate NTS invasiveness. One study examined the *S*. Typhimurium transcriptome under 22 infection-relevant growth conditions involving pathogen stress in the host.[9] RNA-seq has also been used to analyze three outbreak isolates of *S*. Heidelberg under different conditions[10] and an invasive human *S*. Typhimurium ST313 strain.[11] An *in vitro* comparative transcriptome analysis was conducted under oxidative stress without host cells.[12] Transcriptomic changes in *Salmonella* cocultured with macrophages and dendritic cells have also been reported.[7,13–16] However, the characteristics of the altered iNTS transcriptome after the interaction of the pathogens with human intestinal epithelial cells (IECs), the first-line barrier in the gut, remain unexamined. In this study, we used RNA-seq to compare the global transcriptomes of bacteremic and nonbacteremic NTS clinical isolates after their interaction with human IESs as well as of stool-isolated and blood-isolated strains from the same patients.

## 2. Materials and methods

### 2.1. Participants, sample collection, and grouping

From our library of NTS clinical isolates obtained from 98 pediatric patients in the Department of Pediatrics, Shuang Ho Hospital during the period from 2011 until 2019, we selected 24 isolates from 18 patients, whose clinicodemographic characteristics were retrieved from medical records, for analyses (TMU-JIRB No. N201905019). These 24 isolates comprised paired stool and blood isolates of six patients with gastroenteritis and bacteremia (Groups A and B), stool isolates of six patients without bacteremia with C-reactive protein (CRP) levels <1 mg/dL (Group C), and stool isolates of six patients with CRP levels >11 mg/dL (Group D). Among the other 92 patients without bacteremia, the 12 isolates were selected from the 12 patients with the 6 lowest CRP levels and the 6 highest CRP levels among the other 92 patients (Group C + D) that represented NTS not causing bacteremia (Fig. 1).

### 2.2. Bacterial culture and in vitro infection of Caco-2 cells

To obtain the mid-logarithmic cultures ($OD_{600}$ value of 0.7, ~4 × $10^8$ CFU/mL) of the 24 selected NTS isolates, 1:100 dilution of 18-h overnight cultures was prepared and used for *in vitro* infection of Caco-2 cells. *In vitro* NTS infection was mediated by coculturing Caco-2 cells (BCRC No. 67001, originally from American Type Culture Collection No. HTB-37) with each NTS clinical isolate (MOI = 50) for 2 h, as

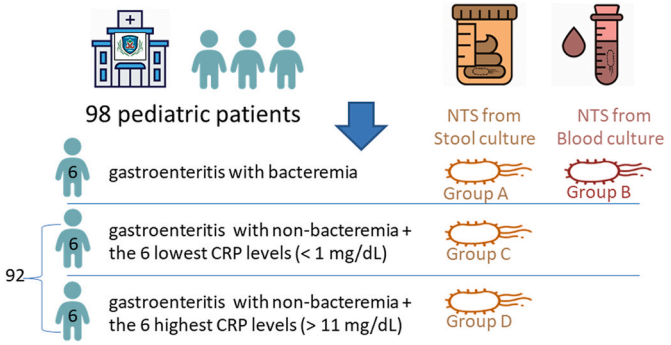



**Fig. 1.** Collection and grouping of the NTS clinical isolates. Among 104 NTS specimens isolated from 98 pediatric patients with nontyphoidal salmonellosis, we collected 24 clinical isolates from 18 patients: isolates from blood cultures (Group A, n = 6) and stool cultures (Group B, n = 6) obtained from six patients with bacteremia; isolates from stool cultures obtained from patients without bacteremia with high CRP levels (Group C, n = 6) or low CRP levels (Group D, n = 6).

previously described.[17] Then, the extracellular bacteria-containing media were collected and centrifuged for harvesting bacterial pellets for subsequent RNA isolation. The confluence of the rest Caco-2 cell monolayers was intact confirmed by Trypan blue staining.

### 2.3. Total RNA isolation and sequencing

Bacterial RNA was isolated using a total RNA isolation kit (Cat #TR01/TR01-150; GeneMark, Taichung, Taiwan) according to the manufacturer's instructions. Residual contaminating genomic DNA was digested using the DNase buffer provided in the kit. Next-generation RNA sequencing was performed using pooled purified RNA samples in the four groups. All procedures were conducted following the Illumina protocol. Gene expression was quantified as reads per kilobase of exon per million mapped reads.[18] The cuffdiff tool from the cufflinks package was used to calculate the expression fold changes and *p* values for each gene between the groups.[19]

### 2.4. Analysis of differentially expressed genes obtained from RNA-seq

The overall difference in gene expression between the four groups was determined using principal component analysis (PCA)[20] and Jensen–Shannon (JS) divergence.[21] Differential expression analysis of individual genes was performed using StringTie (StringTir v2.1.4) and DEseq (DEseq v1.39.0) with genome bias detection/correction and the Welgene Biotech in-house pipeline.[19] In each experiment design, functional enrichment assays of differentially expressed genes (DEGs) were performed using clusterProfiler v3.6. Genes with a low expression level (<0.3 transcripts per million) in any sample were excluded.[22] Differential gene expression was determined for each paired group and was pairwise compared between Group B and Group A or C + D (Tables S1 and S2) and between Group C and Group D. Heat maps were generated to visualize the expression levels of the *Salmonella* pathogenicity island (SPI)-1, SPI-2, and SPI-4 genes.

### 2.5. Identification of core genome

To determine whether our analysis results can represent all NTS strains, the whole genomes of 1278 NTS strains were downloaded from the PATRIC database[23] (Table S3), and a core genome including 2411 genes was generated from the constructed pan-genome.[24] After excluding identical annotations, 2411 gene names are listed in Table S4.

### 2.6. Quantitative real-time reverse transcription polymerase chain reaction

To validate the results of RNA-seq, we performed quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) for 15 significant DEGs. A total RNA sample (4 μg) was reversely transcribed using the iScript cDNA Synthesis Kit according to the manufacturer's instructions (Cat #1708890; BioRad, CA, USA). Primers specific to four upregulated, four downregulated, and seven pathway-involved genes were designed (Table S5). By using the BioRad C100 Real-Time PCR System, 0.1 μg of cDNA was amplified by using iQTM SYBR Green Supermix (2×) (BioRad). The mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Fig. 2A–B).

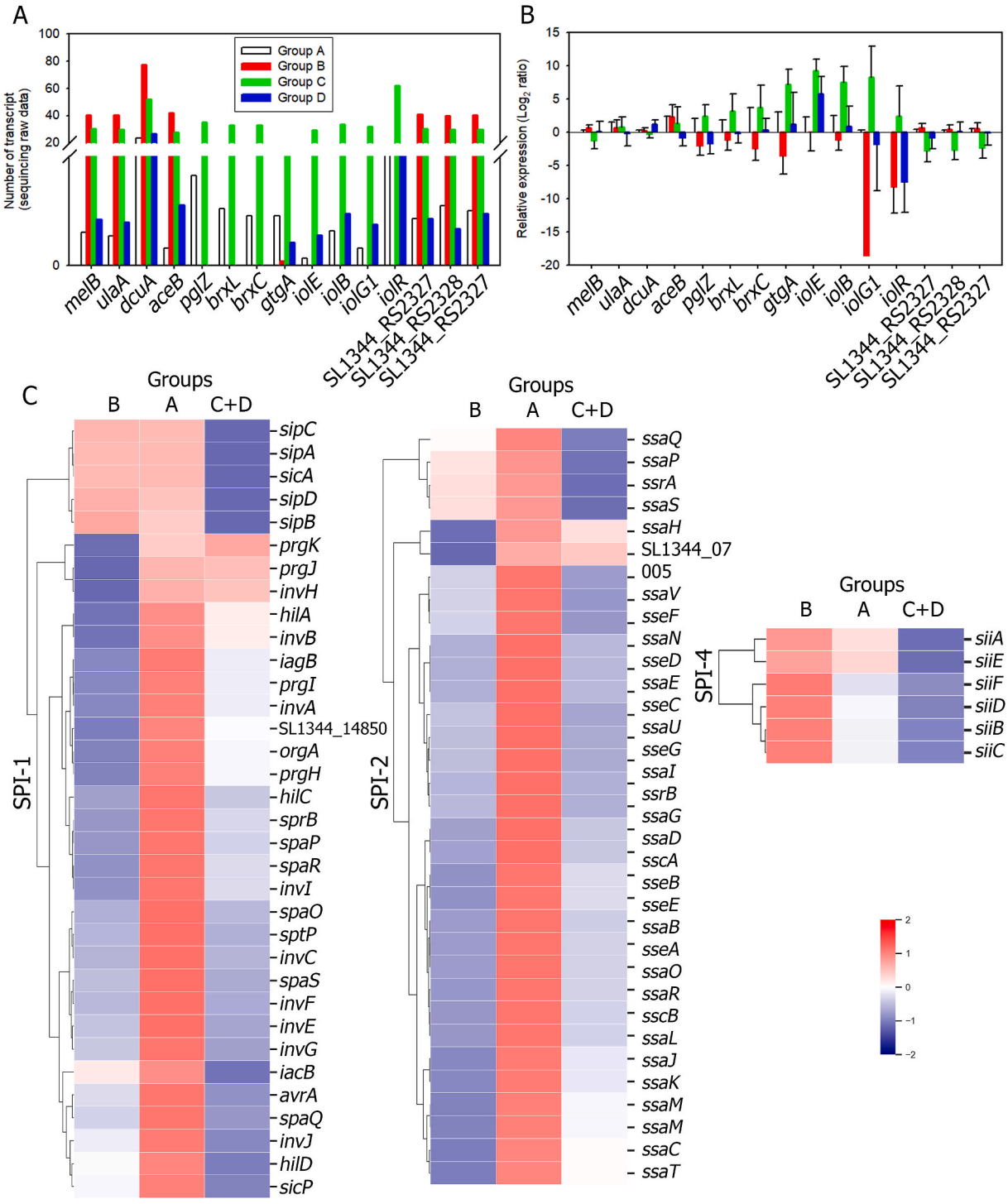



**Fig. 2.** RNA-seq data were validated by qRT-PCR and illustrated using heat maps. Fifteen genes, including the four upregulated genes (*melB*, *ulaA*, *dcuB*, and *aceB*), four downregulated genes (*pglZ*, *brxL*, *brxC*, and *gtgA*), and seven pathway-involved genes (*iolE*, *iolB*, *iolG1*, *iolR*, SL1344_RS23275, SL1344_RS23285, and SL1344_RS23270), were selected for qRT-PCR validation. (A) Raw data of RNA sequencing. (B) Relative expression of the transcripts by qRT-PCR. (C) Heat maps were generated by log2 relative expression of SPI-1, SPI-2, and SPI-4 genes in Group B, Group A and Group C + D.

### 2.7. Gene Ontology term and Kyoto Encyclopedia of Genes and Genomes pathway analyses

This study conducted Gene Ontology (GO) term analysis to investigate the functional clusters of genes involved in NTS bacteremia and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to predict the cellular pathways involved in NTS bacteremia. The enriched GO terms, KEGG pathways, and differential expression of annotated genes are presented in Tables S6 and S7. The DEGs, GO terms, and KEGG pathway analysis are illustrated in terms of Venn diagram, barplots, cnetplots, and emapplots.

### 2.8. Statistical analysis

Clinical variables are expressed as number (percentage) or median [interquartile range]. Between-groups differences in variables were assessed using the chi-square and Kruskal–Wallis tests. Pairwise group differences were calculated using the Bonferroni test. $p < 0.05$ was regarded as statistically significant. For DEGs in the RNA-seq data, $p < 0.01$ and fold change of $> 2.0$ indicated statistically significant differences; adjusted $p$ value was employed for each analysis. GO terms and KEGG pathways with $p < 0.05$ were regarded as significantly enriched.

## 3. Results

### 3.1. Clinical characteristics, except CRP level, were not significantly different between groups

The clinicodemographic characteristics of the three groups are summarized in Table 1; among them, only the CRP levels were significantly different among the three groups with the median CRP level 1.6 [0.8–2.1] mg/dL in the bacteremia group between 0.7 [0.68–0.75] mg/dL and 17.3 [12.6–25.7] mg/dL in the low CRP level and high CRP level nonbacteremia groups, respectively. Notably, the median age of participants was 1.1 [0.9–1.7] years in the bacteremia group, 2.2 [1.5–2.7] years in the nonbacteremia with low CRP level group, and 3.4 [2.9–6.7] years in the nonbacteremia with high CRP level group.

**Table 1**
Clinicodemographic characteristics of the 18 enrolled patients with nontyphoidal salmonellosis.

| Characteristics | Bacteremia group (n = 6) | Nonbacteremia with low CRP group (n = 6) | Nonbacteremia with high CRP group (n = 6) | *p* value |
|---|---|---|---|---|
| *A. Demographic information* | | | | |
| Age in years | 1.1 [0.9–1.7] | 2.2 [1.5–2.7] | 3.4 [2.9–6.7] | 0.80 |
| Sex, male, n (%) | 2 (33.3) | 5 (83.3) | 4 (66.7) | 0.67 |
| Fever, n (%) | 5 (83.3) | 5 (83.3) | 6 (100) | 0.22 |
| Vomiting, n (%) | 1 (16.7) | 2 (33.3) | 3 (50) | 0.52 |
| Diarrhea, n (%) | 6 (100) | 6 (100) | 6 (100) | 1 |
| Bloody stool, n (%) | 4 (66.7) | 5 (83.3) | 5 (83.3) | 0.76 |
| *B. Laboratory data* | | | | |
| Fecal leukocytes, n (%) | 2 (33.3) | 3 (50) | 4 (66.7) | 0.56 |
| Mucus in stool, n (%) | 3 (50) | 2 (33.3) | 4 (66.7) | 0.56 |
| WBC count ($10^3$/μL) | 10.5 [8.1–14.2] | 6.3 [5.8–11.8] | 9.1 [7.3–11.6] | 0.96 |
| NLR ratio | 1.5 [0.9–3.0] | 2.1 [1.6–3.7] | 4.4 [3.6–5.3] | 0.58 |
| Bandemia, n (%) | 1 (16.7) | 1 (16.7) | 2 (33.3) | 0.76 |
| CRP level (mg/dL) | 1.6 [0.8–2.1] | 0.7 [0.68–0.75] | 17.3 [12.6–25.7] | 0.03 |
| *C. NTS serogroup* | | | | |
| Serogroup B, n (%) | 1 (16.7) | 2 (33.3) | 0 (0) | 0.34 |
| Serogroup C, n (%) | 3 (50) | 1 (16.7) | 1 (16.7) | 0.37 |
| Serogroup D, n (%) | 1 (16.7) | 2 (33.3) | 3 (50) | 0.52 |
| Serogroup E, n (%) | 1 (16.7) | 0 (0) | 2 (33.3) | 0.34 |

CRP, C-reactive protein.

### 3.2. Transcripts were differently expressed between the blood-derived and stool-derived NTS strains

PCA and JS divergence indicated that the overall expression of the transcriptomes in Groups A, C, and D was similar, but the transcriptome of Group B was differently expressed from those of the other three groups (Fig. 3A–C). In addition, no remarkable differences were observed in RNA expression between Groups C and D (Table 2). Therefore, the RNA-seq data of Groups C and D were combined to generate a new group, namely Group C + D, which was used for subsequent analyses (Fig. 3D).

The top 10 upregulated and downregulated genes in Group B compared with Groups A, and those in Group B compared with Group C + D are presented (Tables 3 and 4).

To elucidate the altered expression of the *Salmonella* Pathogenicity Island (SPI)-1, SPI-2, and SPI-4 genes in different clinical settings, we investigated their expression levels in the groups that showed remarkable differences between bacteremic blood strains and nonbacteremic strains. SPI-1 genes *sipC*, *sipA*, *sicA*, *sipD*, and *sipB* were upregulated in Group B but downregulated in Group C + D; conversely, *prgK*, *prgJ*, and *invH* were downregulated in Group B but upregulated in Group C + D. SPI-2 genes *ssaP*, *ssrA*, and *ssaS* remained upregulated in Group B but downregulated in Group C + D; oppositely, *ssaH* and SL1344_07005 were downregulated in Group B but upregulated in Group C + D. Notably, all six SPI-4 genes (*siiA*, *siiB*, *siiC*, *siiD*, *siiE*, and *siiF*) were considerably upregulated in Group B compared with Group A, whereas they were significantly downregulated in Group C + D (Fig. 2C).

### 3.3. GO terms and KEGG pathways differed between blood- and stool-isolated NTS strains after Caco-2 cell priming

The significantly enriched GO terms and KEGG pathways in Group B compared with Groups A and C + D are presented using Venn diagrams (Fig. 4A–B). The GO terms related to hydrolase activity, carbohydrate derivative metabolic process, phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), integral component of membrane, and carbohydrate derivative binding; and the KEGG pathways for ascorbate and aldarate metabolism, arginine biosynthesis, PTS, biosynthesis of secondary metabolites, carbon metabolism, sulfur metabolism, and nitrogen metabolism were significantly enriched in Group B only compared with Group A.

We subsequently focused on comparing Group B with Group A because different transcriptomes in the same strains isolated from different host sites can explain the alteration in functional genes related to bacteremia after host immunity priming. The enriched GO terms and KEGG pathways are presented in Tables 5 and 6 and Figs. S1A–B, and the relationships between them are illustrated in Fig. 4B–C and Figs. S1C–D.

### 3.4. Arginine biosynthesis, ascorbate and aldarate metabolism, and PTS pathways were activated in bacteremic blood-derived NTS strains after Caco-2 cell priming

Regarding the enriched KEGG pathways in Group B compared with Group A, the results revealed that ascorbate and aldarate metabolism and PTS were activated by the upregulated *ula* gene cluster. In addition, arginine biosynthesis was activated by the upregulated arg gene cluster. These enriched KEGG pathways are summarized in Fig. 5. All the genes

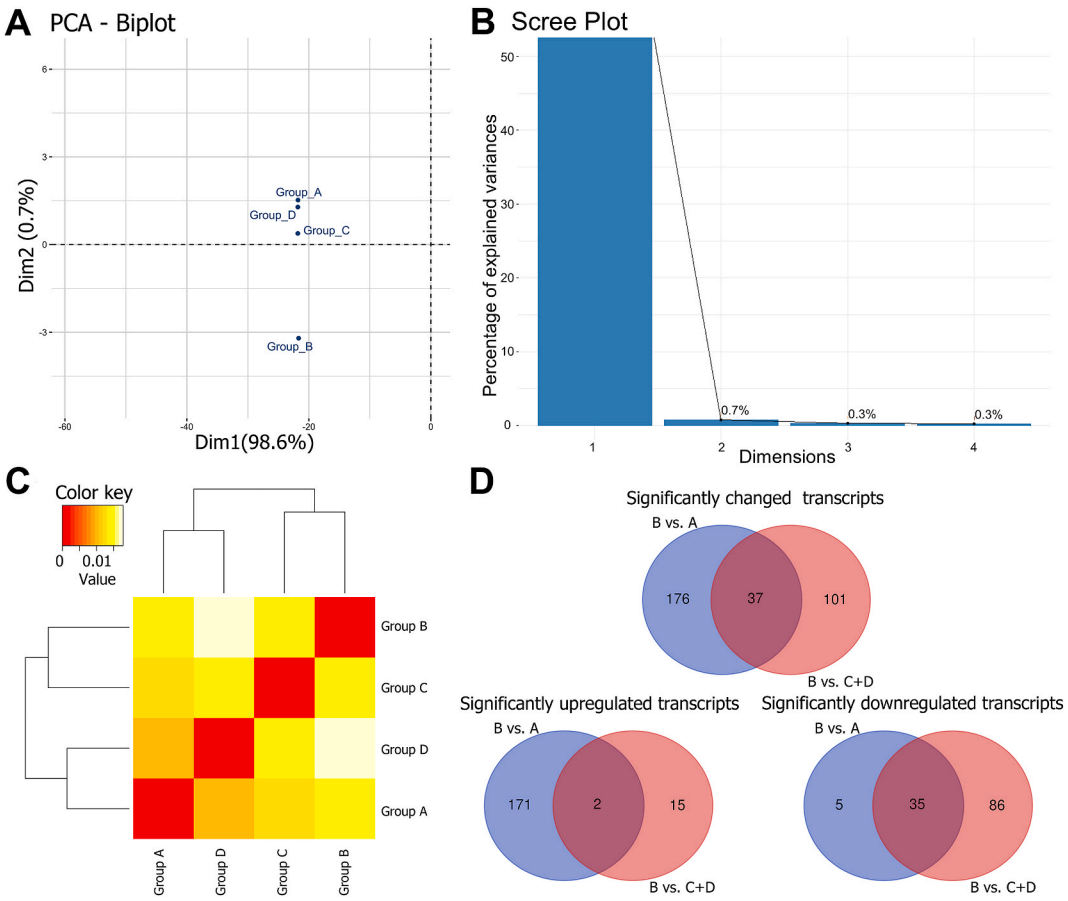



**Fig. 3.** Transcripts of NTS strains collected from blood were differentially expressed compared with those from other groups. Overall difference of transcriptomes among the four groups calculated using PCA is presented using a biplot (A), a Scree plot (B), and by Jensen-Shannon divergence (JSD) is presented using a distance map with color key according to the destandardized values of JS distance (C). Venn diagrams (D) illustrate the numbers of genes with significant changes in expression in Group B compared with Groups A and C + D.

**Table 2**
DEGs, enriched GO terms, and KEGG pathways in three pairwise group comparisons.

| Groups for comparison | No. of significantly[a] upregulated genes | No. of significantly[a] downregulated genes | No. of significantly[a] changed genes | No. of significantly[b] enriched GO terms | No. of significantly[b] enriched KEGG pathway |
|---|---|---|---|---|---|
| B vs. A | 173 | 40 | 213 | 7 | 10 |
| B vs. C + D | 17 | 121 | 138 | 7 | 4 |
| C vs. D | 190 | 12 | 202 | 0 | 6 |

[a] $p << 0.01$ and fold change of >2.0 with DEG analysis.
[b] $p < 0.05$ with GO term and KEGG pathway analysis.

identified in our analysis were confirmed in the core genome of NTS, including the most upregulated genes and approximately half of the downregulated genes.

## 4. Discussion

NTS bacteremia is established after NTS colonizes and invades the intestinal epithelia, enters the bloodstream, and eventually survives after overcoming the host's innate immune response. The bacteria–host interactions during each stage of NTS infection may reveal individual enhanced or weakened biomarkers in either the bacteria (e.g., virulence genes) or the host (e.g., altered characteristics of immune cells), which can be detected using appropriate methods. In this study, we focused on the early priming effect of human IECs on NTS for the subsequent development of bacteremia. We compared global transcriptomes between bacteremic and nonbacteremic NTS clinical isolates after their interaction with Caco-2 cells *in vitro*. Different NTS serovars may differently respond to host cells, with varying adaptive mechanisms. To determine common and key genes or pathways related to bacteremia in NTS clinical isolates, we randomly selected six representative bacteremia-related isolates and compared their transcriptomes with nonbacteremia transcriptomes. Our results revealed that the transcripts of bacteremic blood-derived NTS strains were expressed differently compared with those of stool-derived NTS strains in the other groups. The differentially expressed RNA levels of these transcripts in our *in vitro* model were totally different from previous studies using different cell types and conditions.[9–16] These differently expressed genes could be potential predictive biomarker and treatment targets of *Salmonella* bacteremia.

The CRP is an acute marker of inflammation,[25] and its level is elevated in patients with severe inflammatory disorders. It may protect against invasive *S*. Typhimurium infection in mice.[26] Assuming that CRP levels are associated with bacteremia, we selected NTS clinical isolates from pediatric patients without bacteremia and classified the isolates

**Table 3**
Top 10 significantly upregulated and downregulated transcripts in Group B relative to Group A.

| *S.* Typhimurium locus tag | | Gene description | Log2 fold change | Presence in core genome |
|---|---|---|---|---|
| SL1344 | LT2 | | | |
| Upregulated | | | | |
| *metA* | *metA* | homoserine O-succinyltransferase | 18.68 | Yes |
| SL1344_RS21575 | STM4203 | baseplate protein | 7.62 | ND |
| SL1344_RS21655 | STM4218 | hypothetical protein | 6.45 | ND |
| SL1344_RS21650 | STM4217 | lytic murein transglycosylase | 6.26 | Yes |
| SL1344_RS21545 | STM4197 | putative inner membrane protein | 6.17 | Yes |
| SL1344_RS21570 | STM4202 | baseplate protein | 6.14 | ND |
| SL1344_RS21565 | STM4201 | phage tail protein I | 5.91 | No |
| SL1344_RS21540 | STM4196 | hypothetical protein | 5.76 | ND |
| *aceB* | *aceB* | malate synthase A | 5.73 | No |
| SL1344_RS22900 | STM4464 | ion transporter superfamily protein | 5.63 | ND |
| Downregulated | | | | |
| SL1344_RS20615 | STM4013 | hypothetical protein | −16.97 | ND |
| SL1344_RS23050 | STM4494 | sugar/spermidine/putrescine ABC transporter ATP-binding protein | −16.85 | Yes |
| *iolR* | STM4417 | MurR/RpiR family transcriptional regulator | −16.56 | No |
| SL1344_RS00810 | STM0159 | putative restriction endonuclease | −16.30 | No |
| SL1344_RS24250 | *yigN* | putative inner membrane protein | −16.30 | Yes |
| SL1344_RS19805 | STM3846 | putative reverse transcriptase | −15.69 | No |
| SL1344_RS23030 | STM4490 | putative Mrr restriction endonuclease | −15.56 | No |
| SL1344_RS19800 | STM3845 | hypothetical protein | −15.53 | ND |
| SL1344_RS23040 | STM4492 | putative cytoplasmic protein | −15.32 | No |
| SL1344_RS23025 | STM4489 | DNA helicase | −15.18 | Yes |

ND, not determined due to hypothetical function of genes.

into two groups by CRP levels. Our findings indicated that the median CRP value in the bacteremia group was significantly different from that in the nonbacteremia with high CRP group. This finding is consistent with that a host response leading to high CRP levels might decrease the risk of NTS invasion into the bloodstream.[26] In addition, our RNA-seq analysis revealed no remarkable difference in the transcriptomes of the NTS strains between the high and low CRP groups. In parallel, a recent study in children revealed association of NTS bacteremia with a longer duration of fever and a lower hemoglobin level, but not elevated CRP levels.[27] The CRP levels of patients with bacteremia might be more strongly affected by complex host immune responses than by the transcriptomes of bacteremia-related NTS strains. Therefore, we combined the transcript data of Groups C and D by calculating the mean values of all transcripts. We observed that the transcripts were differently expressed, and that the GO terms and KEGG pathways were differently enriched between bacteremic blood-derived NTS strains and the other groups of strains. A three-dimensional intestinal tissue model with *Salmonella* infection for dual transcriptome sequencing analysis reported the top 10 upregulated genes and downregulated genes.[28] However, these genes were consistently regulated, but not significantly altered, in our RNA-seq data.

SPI-1 genes are generally responsible for bacterial invasion into nonphagocytic cells such as IECs and uptake into phagocytic cells in the early phase of infection.[29] By contrast, SPI-2 genes are required for intracellular survival and bacterial evasion of the oxidase defense system in host cells in the systemic phase of salmonellosis.[30] SPI-1 and SPI-4 genes regulate the interaction of *S*. Typhimurium with the intestinal

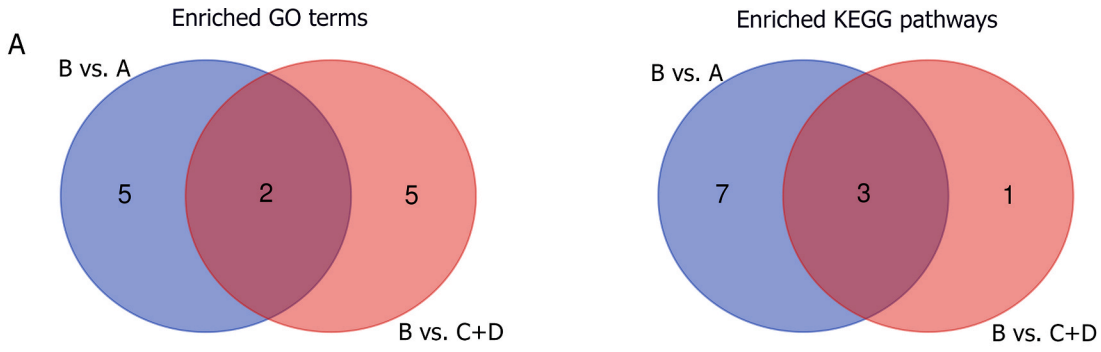
**Table 4**
Top 10 significantly upregulated and downregulated transcripts in Group B compared with Group C + D.

| *S.* Typhimurium locus tag | | Gene description | Log2 fold change | Presence in core genome |
|---|---|---|---|---|
| SL1344 strain | LT2 strain | | | |
| Upregulated | | | | |
| SL1344_RS13405 | STM2340 | putative transketolase | 17.22 | ND |
| *exc* | *putA* | trifunctional transcriptional regulator/proline dehydrogenase/L-glutamate gamma-semialdehyde dehydrogenase | 16.98 | Yes |
| SL1344_RS14325 | STM2767 | DNA helicase | 16.38 | Yes |
| SL1344_RS14320 | STM2766 | hypothetical protein | 16.23 | ND |
| SL1344_RS13265 | STM0926 | putative Fels-1 prophage minor tail protein | 15.67 | No |
| SL1344_RS19470 | STM3782 | PTS galactitol transporter subunit IIC | 15.39 | No |
| *sopE* | *sopE2* | type III secretion protein SopE2 | 15.26 | No |
| SL1344_RS18210 | STM3531 | dihydroxy-acid dehydratase | 15.00 | Yes |
| *lpfC* | *lpfC* | fimbrial assembly protein | 13.45 | Yes |
| *fxsA* | *fxsA* | suppresses F exclusion of bacteriophage T7 | 13.08 | Yes |
| Downregulated | | | | |
| *iolR* | STM4417 | MurR/RpiR family transcriptional regulator | −18.40 | No |
| SL1344_RS23050 | STM4494 | sugar/spermidine/putrescine ABC transporter ATP-binding protein | −17.80 | Yes |
| SL1344_RS04965 | *amiC* | N-acetylmuramoyl-L-alanine amidase | −17.56 | Yes |
| *thiS* | STM4161 | sulfur carrier protein ThiS | −17.54 | No |
| *iolI1* | STM4427 | putative endonuclease | −17.50 | No |
| SL1344_RS23030 | STM4490 | putative Mrr restriction endonuclease | −17.46 | No |
| *iolB* | STM4420 | 5-deoxy-glucuronate isomerase | −17.45 | No |
| SL1344_RS22180 | STM4320 | MerR family transcriptional regulator | −17.44 | Yes |
| SL1344_RS23065 | STM4497 | hypothetical protein | −17.42 | ND |
| SL1344_RS23040 | STM4492 | putative cytoplasmic protein | −17.42 | Yes |

ND, not determined due to hypothetical function of genes.

mucosa in a coordinated manner.[31] Our RNA-seq data demonstrated that most SPI-1 and SPI-2 genes were downregulated in bacteremic blood-derived NTS strains than in bacteremic stool-derived NTS strains; however, SPI-4 genes were all significantly upregulated. SPI-4 genes may contribute more than SPI-1 and SPI-2 genes to *Salmonella* bacteremia during the early interaction between NTS and IECs. Interestingly, a small number of certain SPI-1 genes (*sipC*, *sipA*, *sicA*, *sipD*, and *sipB*) and SPI-2 genes (*ssaP*, *ssrA*, and *ssaS*) remained upregulated in bacteremic blood-derived strains but significantly downregulated in nonbacteremic strains after interacting with Caco-2 cells, suggesting their potential roles in *Salmonella* bacteremia. Thus, our findings provide novel insights into the altered expression of these SPI genes in NTS bacteremia, which occurs as these bacteria pass through the intestinal epithelial barrier and cause a systemic infection. This warrants further in vivo and clinical studies for validation.

In a previous study, a dual transcriptome analysis was conducted to examine the influence of pathogen transcriptomes on the host immune




| | | |
|---|---|---|
| 1. Hydrolase activity<br>2. Carbohydrate derivative metabolic process<br>3. Phosphoenolpyruvate-dependent sugar PTS<br>4. Integral component of membrane<br>5. Carbohydrate derivative binding | 1. Carbohydrate transport<br>2. Endonuclease activity | 1. Fimbrial usher porin activity<br>2. Manganese ion binding<br>3. Pilus assembly<br>4. Transmembrane transporter activity<br>5. Symporter activity |

| | | |
|---|---|---|
| 1. Ascorbate and aldarate metabolism<br>2. Arginine biosynthesis<br>3. Phosphotransferase system<br>4. Biosynthesis of secondary metabolites<br>5. Carbon metabolism<br>6. Sulfur metabolism<br>7. Nitrogen metabolism | 1. Inositol phosphate metabolism<br>2. Streptomycin biosynthesis<br>3. Microbial metabolism in diverse environments | 1. Salmonella infection |

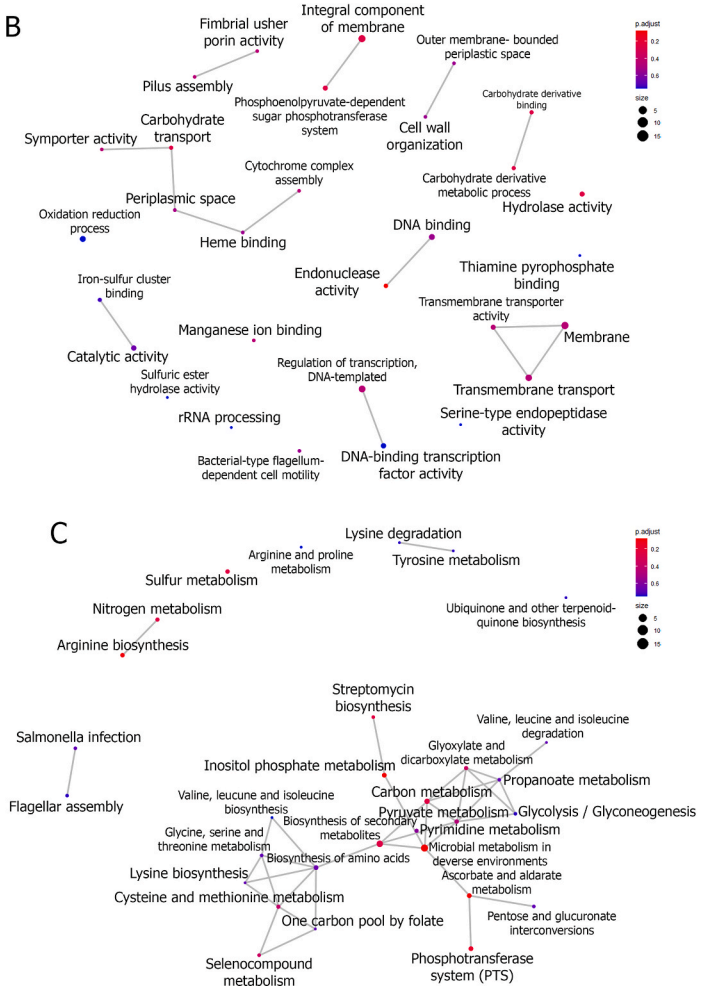



**Fig. 4.** GO terms and KEGG pathways were differentially enriched in NTS strains isolated from blood compared with those in other groups. (A) Venn diagrams reveal the number of enriched GO terms (left diagram) and KEGG pathways (right diagram) in Group B compared with Group A and C + D. (B) Emapplots of enriched GO terms and (C) KEGG pathways indicate the particular GO terms and KEGG pathways sharing common annotated genes and their connections. Dot size indicates the number of significantly changed annotated genes, dot color indicates the significance of enrichment, and lines between dots indicate the sharing of an annotated gene for particular GO terms and KEGG pathways.

**Table 5**
Significantly enriched GO terms detected in Group B compared with Group A.

| GO terms | *p* value | Upregulated genes | Downregulated genes | Number of present genes in core genome | |
|---|---|---|---|---|---|
| | | | | Upregulated | Downregulated |
| Endonuclease activity | 0.0013 | *hsdR*/SL1344_RS23230 | SL1344_RS00810/ SL1344_RS23030 | 0/2 | 0/2 |
| Carbohydrate transport | 0.0170 | *malM/melB* | SL1344_RS20640 | 1/2 | 0/1 |
| Integral component of membrane | 0.0223 | SL1344_RS21590/SL1344_RS21910/*melB/dcuB*/SL1344_RS22115/ *dcuA/ulaA*/SL1344_RS22560/SL1344_RS22585/*iolT1/iolT2*/ SL1344_RS22900/SL1344_RS23265/SL1344 RS23270/ SL1344_RS23280/SL1344_RS23285/SL1344_RS24250 | SL1344_RS20640 | 5/6 | 0/0 |
| Phosphoenolpyruvate-dependent sugar PTS | 0.0226 | *ulaA*/SL1344_RS23265/SL1344_RS23270/SL1344_RS23275/ SL1344_RS23280/SL1344_RS23285 | – | 10/14 | 2/4 |
| Hydrolase activity | 0.0226 | SL1344_RS22600/SL1344_RS22650/SL1344_RS22805/ SL1344_RS23115/SL1344_RS23150/hsdR | – | 5/6 | 0/0 |
| Carbohydrate derivative binding | 0.0302 | SL1344_RS23290/SL1344_RS23295 | *iolR* | 1/2 | 0/1 |
| Carbohydrate derivative metabolic process | 0.0302 | SL1344_RS23290/SL1344_RS23295 | *iolR* | 1/2 | 0/1 |

**Table 6**
Significantly enriched KEGG pathways in Group B compared with Group A.

| KEGG pathways | *p* value | Upregulated genes | Downregulated genes | Number of present genes in core genome | |
|---|---|---|---|---|---|
| | | | | Upregulated | Downregulated |
| Inositol phosphate metabolism | <0.0001 | – | *iolB/iolA/iolG1/iolI1/ iolD/iolG2* | 0/0 | 0/6 |
| Microbial metabolism in diverse environments | 0.0002 | *aceB/aceA/lysC/acs/nrfA*/SL1344_RS22080/*ulaG/ulaA/ulaC/ ulaD/ulaE/arcC/idnK*/SL1344_RS23010/SL1344_RS23190 | *iolB/iolG1/iolI1/iolDiolG2* | 9/15 | 0/5 |
| Ascorbate and aldarate metabolism | 0.0006 | *ulaG/ulaA/ulaC/ulaD/ulaE* | – | 4/5 | 0/0 |
| Arginine biosynthesis | 0.0021 | *argF/arcC/arcA/argF* | – | 3/4 | 0/0 |
| Phosphotransferase system (PTS) | 0.0103 | *ulaA/ulaC*/SL1344_RS23270/SL1344_RS23275/ SL1344_RS23280/SL1344_RS23285 | – | 5/6 | 0/0 |
| Carbon metabolism | 0.0258 | *aceB/aceA/acs*/SL1344_RS22080/*arcC/idnK* | *ilvA/iolA* | 3/6 | 1/2 |
| Sulfur metabolism | 0.0318 | *metA*/SL1344_RS22105/dmsB/SL1344_RS22115 | – | 4/4 | 0/0 |
| Nitrogen metabolism | 0.0357 | *nrfA/arcC* | SL1344_RS23960/ | 0/2 | 1/1 |
| Streptomycin biosynthesis | 0.0390 | – | *iolG1/iolG2* | 0/0 | 0/2 |
| Biosynthesis of secondary metabolites | 0.0461 | *metA/aceB/aceA/metH/lysC/acs*/SL1344_RS22080/ SL1344_RS22590/*argF/arcA/argF/idnK*/SL1344_RS23010 | *ilvA*/SL1344_RS20610/ *iolG1/iolG2* | 11/13 | 2/4 |

response.[28] Our GO term and KEGG pathway analysis of the RNA-seq data revealed that the arginine biosynthesis, ascorbate and aldarate metabolism, and PTS pathways were activated in bacteremic blood-derived NTS clinical strains after their interaction with Caco-2 cells. The PTS is a major mechanism used by bacteria for the uptake of carbohydrates, particularly hexoses, hexitols, and disaccharides.[32] A study reported the versatility of the PTS for interconnecting energy and signal transduction in response to sugar availability.[33] Our analysis indicated the increased rate of the phosphotransferase-catalyzed reaction of fructoselysine/glucoselysine and reaction of L-ascorbate in bacteremia-related NTS strains. A recent study showed that a *Salmonella* strain with alterations in key PTS pathway genes *ptsI* and *crr* downregulated expression of genes associated with quorum sensing, SPIs, flagella, and PhoPQ regulon.[34] Another study demonstrated that a *ptsI*-deleted *Salmonella* strain exhibited a reduced invasion and replication capacity for macrophages and lower virulence in a mouse colitis model; the expression of SPI-1 and SPI-2 genes and the motility of the altered strain were all downregulated compared with the parent strain.[35] Studies have suggested that the virulence of *Salmonella* strains was associated with mutation or deletion of the genes related to the uptake of glucose, but not other sugars. By contrast, we found that other genes related to sugar (fructoselysine/glucoselysine and L-ascorbate) uptake was upregulated in bacteremia-related NTS strains. Whether only glucose uptake or all sugar uptake pathways are associated with invasion of NTS into the bloodstream warrants further study.

The ascorbate and aldarate metabolism pathway was significantly activated in bacteremic blood-derived NTS strains. The annotated genes of the pathway were partially involved in the PTS pathway. L-ascorbate 6-phosphate enters bacteria through the PTS pathway and subsequently activates the ascorbate and aldarate metabolism pathway. Our above identified specific genes both involved in the PTS pathway and ascorbate and aldarate metabolism are promising targets for prognostic, diagnostic or therapeutic implications.

The arginine biosynthesis pathway was also significantly activated in bacteremic blood-derived NTS strains after their interaction with human IECs. Arginine metabolism mediated through the arginine deiminase pathway has been well studied in diverse microorganisms,[36,37] and it is essential for bacterial survival against host defenses.[38] A recent study reported that the arginine sensor ArgR directly activates the expression of the genes encoding T3SS in the presence of arginine. Exogenously added arginine induces *Escherichia coli* virulence gene expression *in vitro*. Δ*artP*, a mutant deficient in arginine transport, also exhibited decreased virulence gene expression.[39] Overall, arginine biosynthesis may play a key role in NTS invasion into the bloodstream as another potential niche for future clinical implications.

In conclusion, our findings indicate that CRP levels are not associated with bacteremia development. The early activation of arginine biosynthesis, ascorbate and aldarate metabolism, and PTS pathways seem to contribute to the bloodstream invasion of NTS. Both human intestinal epithelial cells and host defenses in the bloodstream are closely involved in regulating the expression of the identified NTS genes in the pathways associated with NTS bacteremia. Our findings provide new insights for

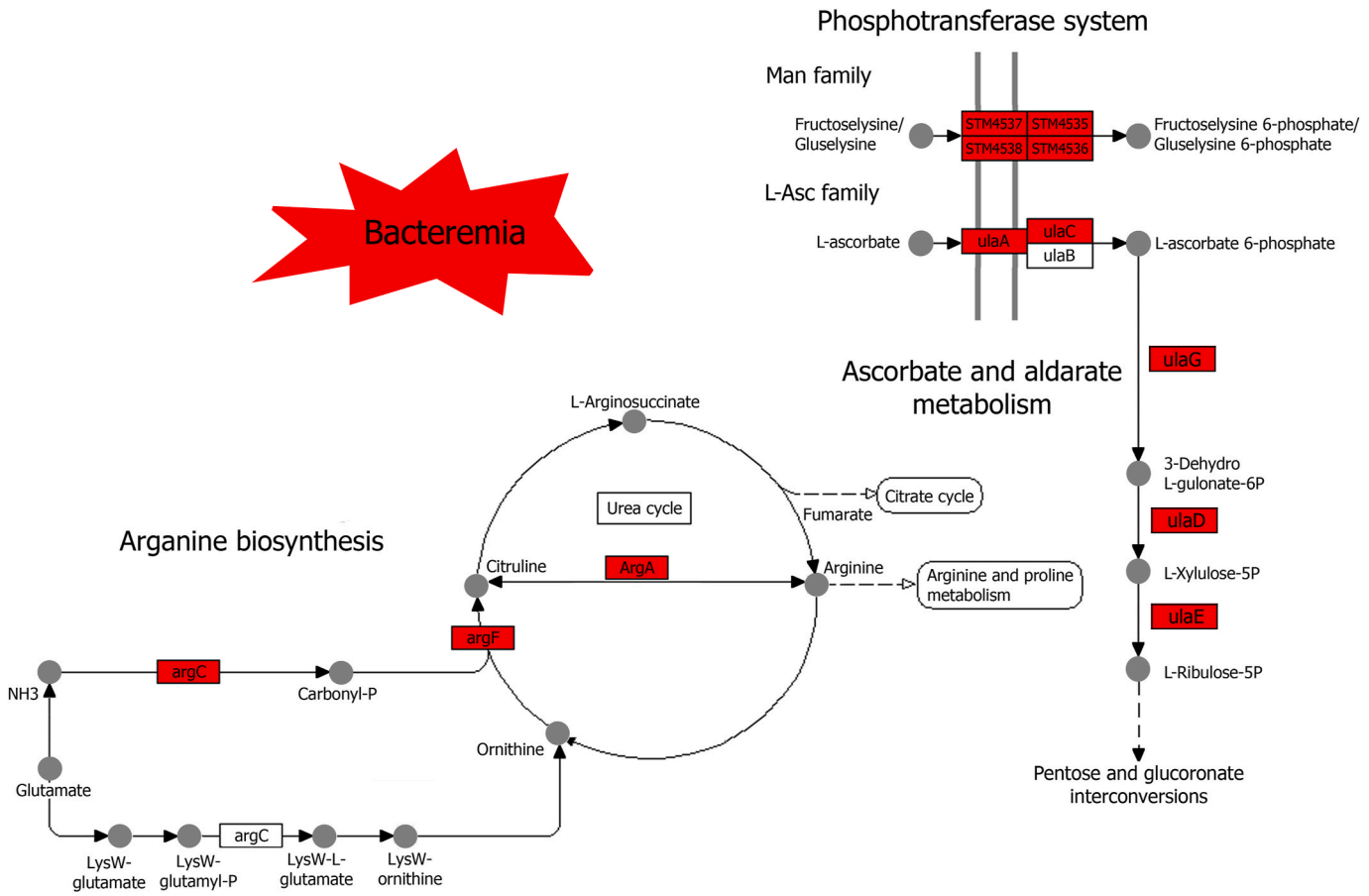



**Fig. 5.** Significantly enriched KEGG pathways with gene clusters in NTS isolates cultured from blood samples after human IEC priming. "Arginine biosynthesis", "ascorbate and aldarate metabolism", and "PTS" pathways were activated. The genes in red color indicate upregulated genes in bacteremic NTS specimens.

the development of predictive biomarkers and early treatment for invasive salmonellosis.

## Data availability

All data generated or analyzed during this study are included in this published article and its supplementary materials.

## CRediT authorship contribution statement

**Buyandelger Batsaikhan:** Writing – original draft, Formal analysis, Data curation. **Pei-Chun Lin:** Writing – original draft, Formal analysis, Data curation. **Katsumi Shigemura:** Visualization, Conceptualization. **Yu-Wei Wu:** Visualization, Formal analysis, Data curation, Conceptualization. **Reo Onishi:** Writing – review & editing, Visualization. **Pei-Ru Chang:** Methodology, Investigation, Formal analysis, Data curation. **Hung-Yen Cheng:** Visualization, Investigation, Data curation. **Shiuh-Bin Fang:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.09.002.

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