SALMONELLA AND MULTIDIRECTIONAL COMMUNICATION IN THE GUT

A Dissertation

by

ELENA VLADIMIROVNA GART

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Chair of Committee,	Sara D. Lawhon
Committee Members,	Jan S. Suchodolski
	Ronald D. Randel
	Robert C. Alaniz
	Thomas H. Welsh, Jr.
Head of Department,	Ramesh Vemulapalli

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ABSTRACT

Salmonella enterica serovar Typhymurium (*S.* Typhimurium) is a bacterial pathogen which is a cause of over a million cases of gastrointestinal illness worldwide. The GI tract is a large and complex environment influenced by both the host and microbes which inhabit the host's gut. In the gut *S.* Typhimurium employs various virulence factors such as SPI-1 and SPI-2 to attach to the epithelial cells and persist in the body. It also initiates host inflammatory responses by inducing production of reactive oxygen species and inflammatory cytokines. Additionally, *S.* Typhimurium uses unique metabolic pathways to compete for limited nutrients under inflammatory conditions and during the initial colonization stage. Some members of the resident microbiota can exacerbate *S.* Typhimurium-induced pathology by providing necessary substrates to the pathogen and by degrading host defense mechanisms. The dense and diverse gut microbiota utilizes a variety of signaling molecules for intra- and inter-species communication to coordinate its members. Resident microbiota can also communicate with the central and enteric nervous system through neural, endocrine, immune and humoral pathways. This brain-gut communication is involved in the regulation of host and microbiota and is greatly affected by stress. While *S.* Typhimurium regulates gene expression by self-produced quorum sensing molecules, such as AI-2 and AI-3, it also recognize signals produced by other microbes and the host in order to regulate its growth and virulence, and in some cases, antimicrobial resistance. In the healthy gut, resident microbiota provides colonization resistance, however inflammation shifts the balance

between the pathogen and microbiota thus contributing to the *S.* Typhimurium blooms. In summary, *S*. Typhimurium employs multiple tactics to establish itself in the gut; however, the microbial composition, and existing inflammatory and neural-hormonal processes also play roles in the development of the *S.* Typhimurium infection. This dissertation discusses the multidirectional interactions of *S.* Typhimurium, host and microbiota.

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Contributors

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NOMENCLATURE

AA	Adrenergic agonist
ACTH	Adrenocorticotropic hormone
AI	Autoinducer
ENS	Enteric nervous system
Epi	Epinephrine
CFU	Colony forming unit
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
DHMA	3,4-Dihydroxymandelic acid
GF	Germ-free
GI	Gastrointestinal
HGT	Horizontal gene transfer
HPA	Hypotalamic-pituitary-adrenal axis
5-HT	5-hydroxytryptamine
IL	Interleukin
KO	KEGG orthology
LB	Lysogeny broth
LPS	Lipopolysacharide
NE	Norepinephrine
R	Ractopamine

ROS	Reactive oxygen species
Rpm	Revolutions per minute
S	Salbutamol
SCFA	Short chain fatty acid
SPF	Specific pathogen-free
SPI	*Salmonella* pathogenicity island
T	Terbutaline
TS	Transconjugant
T3SS	Type 3 secretory system
WT	Wild type

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1. INTRODUCTION*

1.1. *S.* Typhimurium pathogenesis and virulence

Salmonellosis in humans and food animals caused by *S.* Typhimurium is characterized by fever, acute intestinal inflammation and diarrhea within 24 h after infection. *Salmonella* employs multiple virulence factors to overcome colonization resistance and induce inflammation[1]. After entering the intestinal lumen, *Salmonella* uses flagella to move to the proximity of the intestinal epithelial cells, and uses fimbriae for intimate cell attachment (Fig. 1). Fimbriae bind the extracellular matrix glycoprotein laminin and mediate adhesion to the host cell. The autotransporter protein, MisL[2], binds to fibronectin; *Salmonella* adhesins (SiiE and BapA) are also involved in the adhesion process [1]. *Salmonella* pathogenicity islands 1 (SPI-1) and 2 (SPI-2) encode two type III secretory systems (T3SS) which are syringe-like apparatuses which *Salmonella* uses to translocate bacterial proteins into host cells. The SPI-1 T3SS (T3SS-1) is associated with invasion of epithelial cells. Structural proteins build the molecular syringe structure of T3SS. *Salmonella* injects effector proteins SipA, SopA, SopB (SigD), SopD and SopE2 via the needle into the host cell where they trigger cytoskeletal rearrangement and bacterial engulfment (reviewed in detail by[3]).

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Additionally, T3SS-1 effectors induce fluid secretion and promote inflammation[4]. Throughout the invasion process, signaling via pathogen-associated molecular patterns such as flagella and lipopolysaccharide (LPS) induce inflammation. Once inside *Salmonella* - containing vacuoles (SCVs), *Salmonella* induces expression of a second T3SS, encoded on SPI-2, that enables the organism to modify the SCV to withstand oxidative stress and to survive inside macrophages[5]. Concomitant with invasion, epithelial cells, mononuclear cells and complement recognize *Salmonella* and other pathogens and trigger IL-1β, IL-12, IL-18, IL-23, TNF-α, INF-γ and C5a production. These signals instruct the host to implement antibacterial responses including macrophage activation, recruitment of neutrophils, and release of antimicrobial peptides such as α-defensins and cathelicidins by epithelial cells. Activated macrophages and neutrophils release reactive oxygen radicals and antimicrobial peptides that are toxic to commensal microbiota but *S*. Typhimurium detoxifie[6] (Fig. 1).

Therefore, in a hostile take-over, *S*. Typhimurium induces an inflammatory immune response which not only creates new resources like tetrathionate for *S.* Typhimuriun, but also reduces resident microbiota thereby making already existing resources available for *S.* Typhimurium. Hence, the *Salmonella* induces an inflammatory immune response that allows it to compete with commensal microbiota and effectively colonize the gut[1, 7].

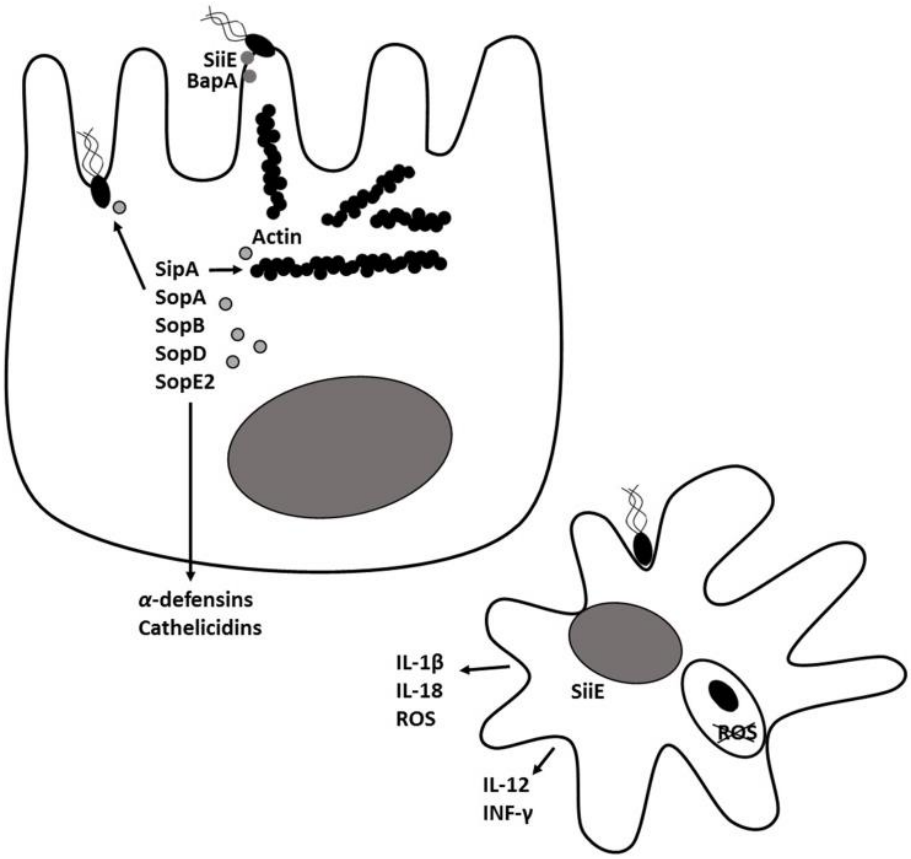

Figure 1: *Salmonella* Typhimurium pathogenesis and virulence. In the intestinal lumen, *Salmonella* uses flagella to move close to the intestinal epithelial cells and uses fimbriae and adhesins (SiiE, BapA) for intimate cell attachment. Through the type III secretion system encoded on pathogenicity island 1 (T3SS-1), *Salmonella* injects effector proteins SipA, SopA, SopB (SigD), SopD and SopE2 into host cells where they trigger cytoskeletal rearrangement, bacterial engulfment and formation of *Salmonella*-containing vacuole (SCV). The T3SS-1 effectors induce secretion of inflammatory cytokines and antimicrobial peptides by epithelial cells. A second type III secretion system encoded on *Salmonella* pathogenicity island 2 (T3SS-2) is expressed within the SCV. Proteins secreted through T3SS-2 prevent production of reactive oxygen species (ROS) and enables *Salmonella* to survive inside macrophages[8].

1.2. Multidirectional signaling in the gut and effect on *S.* Typhimurium

Quorum sensing (QS) is a method of bacterial cell-to-cell signaling which allows the bacteria to coordinate gene expression at the population level. Receptors on bacterial

cells recognize secreted molecules and initiate expression of downstream genes, including those involved in synthesis of QS compounds.

Bacterial signaling was discovered decades ago in the gram negative bacterium *Aliivibrio fischeri*, which inhabits the photophore of Hawaiian bobtail squid. It was determined that when the *A. fisheri* population in the squid's photophore reaches a particular density it bioluminesces. Surprisingly, the growth of *A. fisheri* in spent media resulted in induction of luminescence at lower cell densities[9]. This led to the conclusion that high concentrations of signaling molecules secreted into the media by other cells cause the bacteria to luminesce. Besides intraspecies communication, bacteria are capable of interspecies[10] and interkingdom[11] communication. In the gastrointestinal environment bacteria produce hormones and hormone-like substances[12] or modify the host's signaling molecules, thus, affecting the host[13]. Additionally, interspecies and interkingdom signaling modulate bacterial growth and virulence of pathogenic bacteria[14].

1.3. *S.* Typhimurium autoinducers and role in virulence

1.3.1. AI-2

More than a decade ago Surette *et al*., discovered that *E. coli* and *S.* Typhimurium secrete a small, soluble, heat-labile, signaling molecule, which was named autoinducer-2 (AI-2)[15]. The signal concentration in growth media was maximal at the mid-exponential phase yet disappeared from the media at the beginning of the stationary phase in coordination with glucose depletion[16]. Additionally, AI-2-dependent signaling

required low pH and high osmolality, whereas low osmolality induced signal degradation[17].

AI-2 production in *S.* Typhimurium depends on a series of enzymatic reactions. First, methyl transferases convert S-adenosyl methionine (SAM) to S-adenosylhomocysteine (SAH), then methylthioadenosine/S-adenosylhomocysteine nucleosidase (Pfs) and S-ribosylhomocysteinase (LuxS) convert SAH to 4,5 dihydroxy-2,3-pentanedione (DPD). Finally, DPD is cyclized into (2R, 4S)-2-methyl-2,3,3,4-tetrahydroxytetra-hydrofuran (R-THMF, AI-2)[18]. *S.* Typhimurium produces and releases the majority of the AI-2 during exponential growth[18a] and membrane transport protein YdgG may be involved in the extracellular transport of AI-2[19]. Extracellular AI-2 binds to autoinducer binding protein LsrB and is transported into bacterial cell via the ATP transporter encoded on *lsr* operon by *lsrACDB*[20]. The phosphoenol pyruvate phosphotransferase system (PTS) is essential for initial ABC transporter activation and AI-2 internalization[21]. A cytoplasmic kinase, LsrK, phosphorylates internalized AI-2[18a]. Phosphorylated AI-2 inactivates the transcriptional repressor protein LsrR in a dose-dependent manner[20] and induces *lsr*ACDBFGE operon transcription[18a]. The LsrR protein, encoded on the *lsr* operon, binds two loosely conserved sites on *lsr,* thus, repressing the transcription of *lsr*ACDBFGE and itself[20]. Phospho-AI-2 is degraded by LsrG and LsrF. Since the *lsr* expression is delayed but not completely halted in a *lsrB* mutant, there may be an alternative AI-2 transporter in *S. Typhimurum*[18a]. Transcriptome analysis suggests that *rbsB* gene encoding ABC superfamily D-ribose transport protein may be involved in the AI-2 transport in *S. Typhimurum*[22]. In *S.* Typhimurium, LsrR,

also negatively regulates expression of genes involved in the oxidative stress response (*sodA, sodCI, sodCII*), which, in turn lowers the bacterial ability to survive within macrophages[23]. Additionally, LsrR represses flagella expression and invasion. Inactivation of LsrR by the presence of phosphorylated AI-2 allows SPI-1 (*invF, sicA, sopB, sopE*) and flagella (*fliC, fliD*) gene transcription[24] (Fig. 2).

Although, the *lsr* operon is the only system known to be directly regulated by AI-2 in *E. coli* and *Salmonella*, proteomic analysis revealed that deletion of *luxS* in EHEC affected a variety of cell functions, including cell signaling, metabolism, information storage and processing, possibly through repressed tryptophan biosynthesis[25]. More than 500 genes were differentially expressed between WT and a *luxS S.* Typhimurium mutant *in vitro*[22]. For example, deletion of *luxS* decreased the expression of genes encoding flagellar motility and chemotaxis as well as genes encoded on SPI-1 and *lsr*; however, the expression of *hilD, hilA, sipB* and *invABCE* virulence factors increased[22]. Phenotypically, deletion of *luxS* resulted in a decreased motility and virulence *in vitro*, which was restored by AI-2 supplementation in the media[26]. A *S.* Typhiumurium *luxS* mutant was also defective for virulence in mice and epithelial cell invasion[26]. A combination of signals can modify AI-2 dependent virulence in *S.* Typhimurium. For example, *luxS* mutants have been shown to grow poorly in nutrient-poor M9 minimal medium. The addition of AI-2 restored growth, but resulted in impaired macrophage invasion which could be reversed with long chain fatty acids (linoleic, oleic, palmitic, stearic) supplementation[27]. This connection between AI-2 and nutrient utilization

suggests that nutrient availability in the complex conditions in the gut may affect *S.* Typhimurium signaling and virulence in addition to QS molecules.

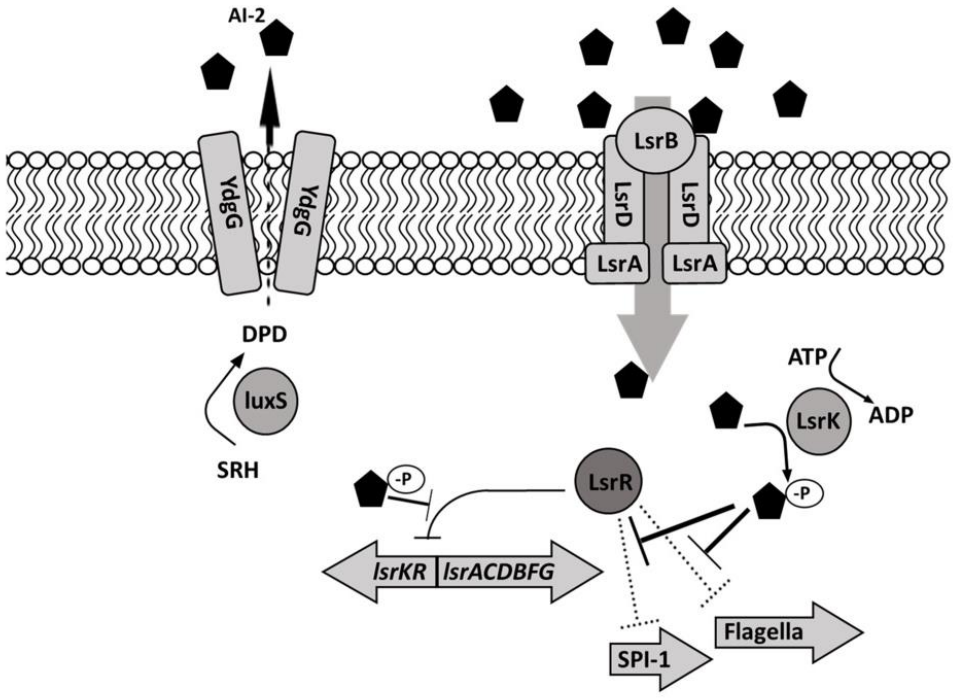

Figure 2: *Salmonella* Typhimurium autoinducer-2 (AI-2) signaling. The signaling molecule AI-2 is produced by LuxS during an exponential growth, is exported by an unknown mechanism (possibly by YdgG), and accumulates extracellularly. Extracellular AI-2 is sensed by the *lsr*-encoded transport cassette and is actively transported into the bacterial cell. Following intracellular phosphorylation by LsrK, AI-2 negatively regulates the transcriptional repressor protein LsrR, allowing the transcription of *lsr*- and SPI-1 – encoded genes as well as flagellar genes[8].

Interestingly, *E. coli* is able to intercept extracellular AI-2 produced by other bacterial species *in vitro*[28] and since microbiota produces AI-2 like molecules[29], it is possible that Enterobacteriaceae can utilize interspecies signaling in the gut. In *E. coli*, chemotaxis to AI-2 depends on the L-serine Tsr receptor and AI-2-binding protein

LsrB[30]. In addition to regulation of virulence, increased concentrations of AI-2 promoted plasmid exchange between 2 different strains of *E. coli*[31] suggesting that AI-2-dependent QS may facilitate genetic information exchange, although this has not been directly shown in *S.* Typhimurium.

1.3.2. AI-3/NE/Epi

Sperandio *et al.* discovered autoinducer 3 (AI-3) production by *Enterobacteriaceae* in 2003; however, the synthetic pathway and chemical formula of AI-3 are still unknown[32]. The two-component regulators associated with AI-3 are associated with recognition of host catecholamines (CA) epinephrine (Epi) and norepinephrine (NE). *Salmonella* and *E. coli* respond to host catecholamines through two-component regulatory systems. *Salmonella* Typhimurium encodes orthologues of the *E. coli* two-component regulatory systems QseC/B (PreB/PreA) and QseF/E[33 34], where QseC and QseE are histidine sensor kinases and QseB and QseF are, respectively, their associated response regulators. QseC is able to sense AI-3 as well as Epi and NE, while QseF can recognize Epi and NE along with sulfate and phosphate[35]. The adrenergic histidine kinase, QseC, autophosphorylates upon binding to AI-3/Epi/NE and then dephosphorylates the response regulator QseB, inducing SPI-2 gene expression[36]. The QseC/QseB two-component regulatory system controls virulence factors such as motility and invasion and survival in macrophages through SPI-1 and SPI-2 encoded virulence genes[36b]. QseE senses NE, Epi, phosphate and sulfate and acts on the response regulator QseF, which induces the expression of SPI-1 encoded genes. The Sperandio group proposed a QseC/QseE interplay model, where QseC phosphorylates QseF, thus

indirectly controlling motility and invasion virulence factors encoded by SPI-1. Overall, it seems that QseE plays an important role during epithelial cell invasion, while QseC is more important in systemic disease and intramacrophage replication[35]. The QseC-dependent signaling can be blocked by the α-adrenergic antagonist phentolamine [37]: however, the effect of adrenergic antagonists on QseE is yet to be determined.

In *Salmonella*, catecholamines induce growth in iron-restricted media[34, 38] and facilitate the expression of genes encoded on SPI-1 (*sipA, sipB*) and SPI-3 (*mgt*) thus modulating virulence of *S.* Typhimurium *in vitro* and *in vivo*[36b]. It is unclear whether NE-dependent enhancement of bacterial growth, motility and virulence depends on QseC/E signaling[34, 36b]. It has been shown that the alpha-adrenergic antagonist, phentolamine, and the beta-adrenergic antagonist, labetalol, can neutralize norepinephrine-induced enhancement of motility[36a, 39] suggesting similarity between bacterial and mammalian targets for adrenergic receptors. It is important to note that the chemoattractant properties of NE are in fact attributed to a bacterial NE metabolite, 3,4-dihydrohymandelic acid (DHMA). When the bacterial cell senses NE via QseC, the cell induces transcription of tyramine oxidase (*tynA*) and aromatic aldehyde dehydrogenase (*feaB*), which are involved in 3,4-dihydroxyphenylglycolaldehyde (DOPEGAL) and DHMA production. The latter is sensed by the bacterial serine chemoreceptor Tsr, which induces concentration-dependent chemotaxis of *E. coli*[40] (Fig. 3). Catecholamines may also play a role in transfer of antimicrobial resistance between *Salmonella* and *E. coli*, as physiological concentrations of NE (5μM) have been shown to enhance plasmid transfer between *S.* Typhimurium and commensal *E. coli* in iron-rich lysogeny broth (LB) broth.

Phentolamine and the beta-adrenergic antagonist, propranolol, inhibited NE-induced plasmid transfer[41]. Finally catecholamine signaling may be involved in AI-2-dependent virulence regulation in *Enterobacteriaceae* as microarray analysis revealed that NE and Epi decrease the expression of *lsr* operon in *E. coli* O157:H7 *in vitro*[42].

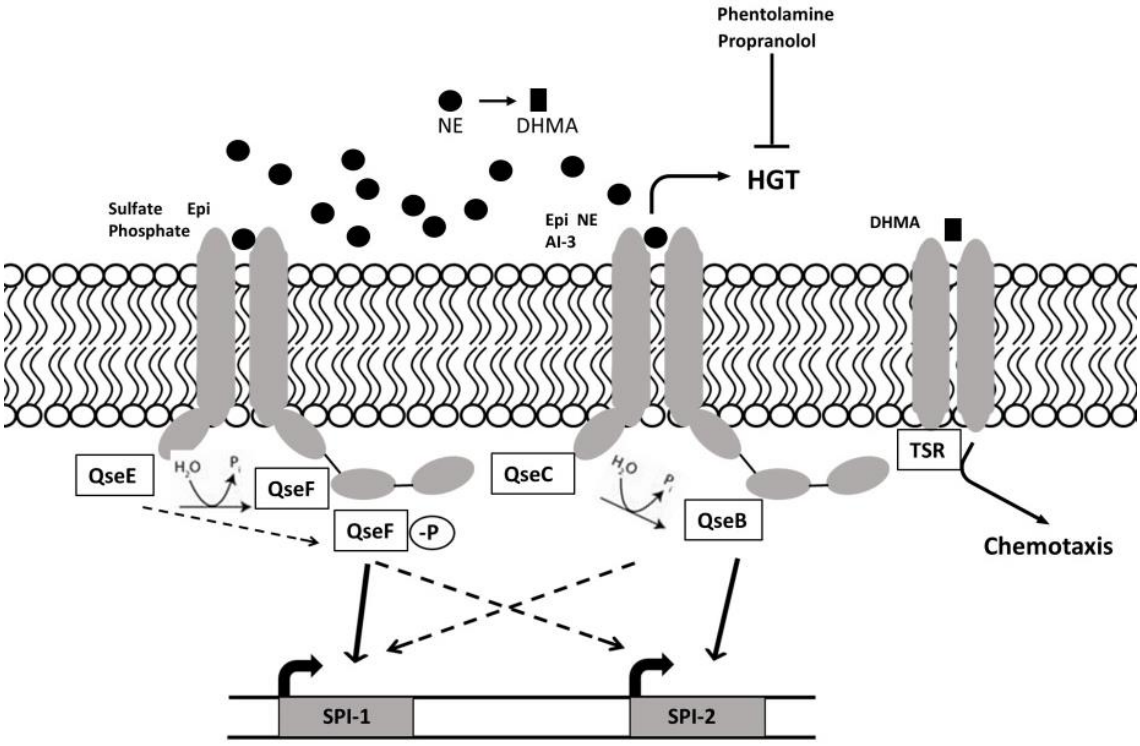

Figure 3: AI-3/NE/Epi quorum sensing in *S*. Typhimurium. Intestinal catecholamines are sensed by *S.* Typhimurium QseC/B and QseE/F regulatory systems. The sensor kinase QseE detects Epi, which leads to phosphorylation of response regulator QseF and subsequent induction of cell invasion genes encoded on SPI-1. Sensor kinase QseC autophosphorylates upon sensing Epi, NE and AI-3, and activates genes encoded by SPI-2 required for intracellular survival) through dephosphorylated response regulator QseB. QseE and QseC indirectly regulate SPI-2 and SPI-1 through QseB and QseF respectively. QseC-dependent increase in horizontal gene transfer and motility can be blocked by adrenergic antagonists phentolamine and propranolol. NE metabolite DHMA is sensed by serine chomoreceptor Tsr and induces chemotaxis[8].

1.4. Interspecies and interkingdom communication

1.4.1. AHL

Several bacterial species such as *Vibrio cholera*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* utilize acyl-homoserine lactone (AHL) signaling[43]. In gram negative bacteria AHL is synthesized by LuxI, which binds to its cognate receptor LuxR, which subsequently binds specific DNA promoters and activates transcription of target genes[11a]. Unexpectedly, in the mammalian gut, AHL was detected only in the bovine and ovine rumens where its concentration was correlated with the summer and spring seasons and *E. coli* O157:H7 colonization, however, it is not known which members of rumen microbiota produce AHL[44 45].

In *S.* Typhimurium AHLs stimulate biofilm formation by regulating glycogen biosynthesis (*glgC*), fimbriae (*fliF*, *lpfA*, and *fimF*) and SPI-1 genes (*hilA* , *invA*, and *invF*)[46]. However, *Salmonella* spp. do not encode AHL synthase and do not produce AHL. Instead, *S.* Typhimurium utilizes the SdiA receptor, which is able to sense AHL produced by other bacterial species[47] when grown on motility agar or during the late exponential phase in liquid culture[48]. Environmental conditions such as low pH, high iron concentrations, or growth in spent media, have been shown to repress *sdiA* expression in *S.* Typhimurium. In mice, infection with a *sdiA* mutant lacking AHL signaling resulted in increased fecal shedding and bacterial loads in the livers of infected mice[49] indicating that SdiA may be a negative regulator of virulence. Together, these data suggest that while no detectable concentrations of AHL have been found in the mammalian gut to date, there may be individual bacteria that secrete small quantities of

AHL. This idea is supported by the finding that the *Salmonella* AHL-receptor SdiA was activated in the GI tract of turtles colonized with *Aeromonas hydrophila*[50]. Alternatively, *S.* Typhimurium may utilize the SdiA sensor kinase for other functions such as the detection of other QS molecules such as indole[51], and to facilitate antimicrobial resistance[52 53].

1.4.2. AI-2/AI-3

In the mammalian gut two dominating phyla, Bacteroidetes and Firmicutes, are predicted to produce the majority (17% and 83% respectively) of AI-2[54]. Within Firmicutes, 97% of Bacilli, 49% of Clostrida and 27% of other taxonomic classes are predicted to have AI-2 production capabilities[54]. Streptomycin has been shown to dramatically shift microbiota composition in the murine gut in the favor of Bacteroidetes[54]. However, introduction of the *E. coli lsrK* mutant, which overproduces AI-2, increased relative abundance of Firmicutes in streptomycin-treated mice, decreasing the Bacteroidetes to Firmicutes ratio[54]. Thus, AI-2 signaling diminishes the effect of streptomycin-induced dysbiosis on microbiota[54]. The microbiota is not the only potential source of AI-2 in the gut. Intriguingly, mammalian cells of epithelial lineage, such as Caco-2 produce a compound which mimics bacterial AI-2 and is detectable by reporter *Vibrio* strain. Increased production of this AI-2 mimic is induced by bacteria, especially when the tight junctions are compromised. This suggests that secretion of signaling molecules, such as the AI-2 mimic by the host allow it to manipulate bacterial behavior in the gut possibly as a protective measure against bacterial transit from the intestinal lumen to the circulatory system[55].

AI-3 signaling has been described in several bacterial species such as *E. coli*, *Salmonella* sp., *Shigella* sp., *Klebsiella pneumoniae* and *Enterobacter cloacae in vitro*[32] and the AI-3 dependent QseC/E system is activated by catecholamines, which are abundant in the mammalian gut[35]. The importance of the AI-3 communication in gastrointestinal dysbiosis has not been extensively investigated but might offer unique opportunities for disease management.

1.4.3. Gut microbiota is involved in the modulation of catecholamines

The majority of CA (NE, Epi, dopamine (DA)) found in blood and urine is conjugated (glucuronide- or sulfate-conjugated) and biologically inactive[56]. Free CA as well as sulfotransferase enzymes, which conjugate free CA, are gradually distributed in the gut with the lowest concentration in the stomach and highest concentration in the large intestine, which correlates with bacterial loads in these organs[13b 57].

It has been recently discovered that the microbiota are involved in the production of biologically active free CAs in the murine intestine. The majority of the CA in the heavily populated cecum and colon of conventional mice were free; while in the sparsely populated ileum a substantial amount of CA was conjugated[13b]. Moreover, the majority of CA in the lumen of germ free (GF) mice was conjugated, and their concentration was lower than in specific pathogen free (SPF) mice. Additionally, β-glucuronidase (GUS) activity, necessary for free CA production, was lower in GF mice. Fecal transplants from SPF mice increased the free CA concentrations and GUS activity in the intestinal lumen of GF mice[13b]. These data indicate the importance of commensal bacteria in GUS-dependent production of biologically active CA in the murine gut.

1.4.4. Iron

Iron is essential for the bacterial cell; however, the majority of extracellular iron in the gut is bound by iron-chelating glycoproteins transferrin and lactoferrin. CA can bind to iron within transferrin and lactoferrin, resulting in release of iron from these complexes followed by their uptake by the bacterial siderophores enterobactin and salmochellin. Porin proteins OmpA and OmpC bring transferrin complexes close to the bacterial cell surface for more efficient uptake of CA-released iron by bacterial siderophores[58]. Porins also allow direct uptake of NE and DA which can enhance bacterial growth[58c]. For *Salmonella*, NE-induced growth requires the catecholate siderophore receptors IroN, FepA and CirA[59] as well as siderophore hydrolysis, which liberates siderophore-bound iron in the cytoplasm[59-60]. However, the catecholate transport systems may not be required for swine colonization by *S.* Typhimurium[36a].The role of QseC/E signaling in NE-dependent bacterial growth needs to be further elucidated[34, 38].

1.4.5. Short chain fatty acids

Microbiota, including pathogenic *E. coli* and *Salmonella* spp. produce short chain fatty acids (SCFA) acetate, propionate and butyrate as the main end-fermentation products that are absorbed by colonic mucosa and used as an energy source by the host and bacteria[61]. In *S.* Typhimurium, SCFAs signal through a two-component regulatory system SirA/BarA primarily at the late exponential phase[62]. BarA is a sensor kinase, which phosphorylates the response regulator SirA in response to extracellular signals. SirA then regulates virulence gene expression through the *csrBC*-CsrA regulatory

cascade[63]. A mixture of SCFAs similar to that found in the distal ileum has been shown to induce the SPI-1 genes *hilA*, *invF*, and *sipC*, while a mixture of SCFAs mimicking the concentration and composition of colonic SCFAs had an opposite effect[62]. Individually, propionate has been shown to decrease SPI-1 expression through the transcriptional regulator HilD[64]. Furthermore, pre-incubation of *S. enteritidis* with propionate and butyrate, but not formate, resulted in decreased epithelial cell invasion[65]. Finally, in pigs, supplementation with a mixture of organic acids including propionate, resulted in decreased *Salmonella* recovery from the mesenteric lymph nodes[66]. Thus supplementation of SCFAs, particularly, propionate, may be a promising intervention strategy for decreasing *Salmonella* loads in farm animals.

1.4.6. Indole secreted by commensal bacteria promotes intestinal health and attenuates S. *Typhimurium virulence*

To date, over 85 bacterial species have been found to produce the small signaling molecule indole. *E. coli* produces indole during the stationary phase with the help of tryptophanase (TnaA), which converts tryptophan into indole, pyruvate and ammonia. Indole is generated exclusively by bacteria but can be absorbed by mammalian hosts; therefore, a number of indole derivatives found in blood are entirely dependent on supply of indole from intestinal microbiota[67]. Indole has been shown to promote the health of the gastrointestinal barrier by increasing transepithelial resistance and decreasing inflammatory cytokine (IL-8, IL-10 and NF-κB) secretion and EHEC attachment[68]. For *E. coli*, indole has been shown to induce SdiA-dependent and temperature-sensitive reduction in biofilm formation and motility[69].

While *Salmonella* does not produce indole, it is able to sense indole produced by other bacterial species through an unknown mechanism. It has been suggested that indole signaling in *E. coli* occurs via the AHL receptor SdiA. Activation of SdiA by indole leads to reduced biofilm formation as well as reduced acid resistance[51]. However, another group determined that an indole associated decrease in biofilm formation in *S.* Typhimurium and *E. coli* was not dependent on SdiA. Instead, high indole concentrations were found to inhibit AHL detection by SdiA[70].

Recent studies have shown that *S.* Typhimurium uses the intercellular signaling molecule indole to increase antibiotic tolerance throughout its population by mediating oxidative stress and phage shock response[52] as well through induction of multidrug efflux pumps[53, 71]. Although indole is the primary metabolite produced from tryptophan by the microbiota, other microbiota-derived tryptophan metabolites exist and likely regulate other microbiota and pathogen properties[42, 51b, 69].

1.4.7. Fucose

Fucose is an abundant sugar in the intestine and its production is microbiota-dependent. For instance *B. thetaiotaomicron* facilitates fucose cleavage from glycans such as mucin[72]. The two-component signal transduction system FusKR of enterohemorrhagic *E. coli* (EHEC) senses fucose and represses LEE expression in the mucus layer, thus preventing energy waste when bacteria are not in close proximity to epithelial cells[73].

In the intestinal lumen, *S.* Typhimurium expresses fucose utilization proteins FucI, FucU and FucA, which are seldom observed *in vitro*[74]. It has been proposed that

intestinal conditions, such as a shift in gut microbiome population, modulate substrate utilization by the pathogen. *S.* Typhimurium exhibited increased expression of genes involved in metabolism of sialic acid, fucose and propanediol in *B. thetaiotaomicron*-monoassociated mice[75]. However, the interplay between fucose sensing and virulence in *S.* Typhimurium is still unknown.

1.5. Gut-brain communication in health and disease

The bidirectional gut-brain communication involves the central (CNS), neuroendocrine, neuroimmune, autonomous and enteric nervous systems (ENS). The neurochemical signals travel from the gut via the afferent neurons while the efferent neurons carry the signals from the brain back to the gut. This cross-talk plays an important role in connecting the central and peripheral nervous systems in order to regulate immunity, digestion and neuroendocrine signaling. Physiological and psychological stressors induce the release of corticotropin-releasing hormone (CRH) from the hypothalamus which stimulates adrenocorticotropic (ACTH) release by the pituitary gland. ACTH stimulates cortisol release from the adrenal glands. Receptors to cortisol (β adrenergic) are present in various tissues and organs including the brain, the gut and the gut microbiota as well[76]. Thus stress stimulates bi-directional communication between the brain and peripheral organs. It has been recently recognized that microbiota are involved in the formation and function of the gut-brain, and, in particular, hypothalamic pituitary axis (HPA), which regulates the response of the host to stress[77]. The catecholamine stress hormone NE is synthesized in the adrenal medulla and postganglionic neurons of the sympathetic nervous system. NE is released in the gut

via the sympathetic nervous system in the longitudinal muscle, on the border of muscularis interna and submucosa, as well as at the junction of interdomal regions of mucosa and submucosa[76]. NE binds to α and β adrenergic receptors when it is at low and high concentrations respectively[76]. NE has segment-specific activity in the intestine. Additionally, NE-mediated epithelial response in the proximal and distal colon can be suppressed by α-blocker phentolamine and β-blocker propranolol respectively[78].

1.5.1. Gut-brain communication: microbiota to host

Neutrotransmitter serotonin (5-hydroxytryptamine, 5-HT), which regulates mood, appetite and sleep is a striking example of a neurochemical which production depends on gut microbiota. The majority (~90%) of 5-HT is synthesized in the gut[79] and is microbiota-dependent, because plasma concentrations of serotonin were almost 3-fold higher in the conventional mice compared to germ-free (GF) animals[67]. Hallmark studies determined that gut microbiota, particularly spore-forming bacteria, modulate intestinal metabolites alpha-tocopherol, butyrate, cholate, deoxycholate, p-aminobenzoate, propionate and tyramine to act directly on enterochromaffin cells, stimulating synthesis of serotonin biosynthetic enzyme, Tph1, thus promoting serotonin synthesis[13a, 80] However, the systemic effect of bacterial regulation of serotonin biosynthesis on distal tissues and organs is unknown[79].

Tryptophan is another example of microbiota-dependent metabolism of signaling molecules. Serum tryptophan concentrations were significantly higher in germ-free mice since they were lacking microbes that convert tryptophan to indole. Additionally, an indole metabolite, indole-3-propionic acid (IPA), has been identified only in plasma of

conventional mice and colonization of GF mice with the commensal bacterium, *C. sporogenes*, restored IPA in the serum of GF mice to the levels found in conventional mice[67].

Sex hormones have been implicated in the modulation of the brain-gut axis at the CNS, autonomous NS and ENS levels with certain differences seen between males and females in pain perception and HPA response. Brain-microbiota communication may also be modulated by sex hormones[81], while bacteria can synthesize steroid metabolizing enzymes thus modulating the host[82]. For example, microbial β-glucuronidase deconjugates estrogens in the gut and makes them available for reabsorption via enterohepatic circulation[83] and regulates testosterone production[84]. Additionally, *S.* Typhimurium has been found to alter multiple host metabolic pathways, particularly, steroid hormones[85]. Finally, the microbiota is required for biologically active CA production (NE, Epi and dopamine) in the intestinal lumen, which can be utilized by the host and microbiota (Asano et al., 2012). On the other hand estrogen receptor β (ERβ) status affects species richness and relative abundance of prominent phylotypes of murine fecal microbiota composition, where relative abundance of Bacteroidetes and Proteobacteria was higher in $ER\beta^{+/+}$ $ER\beta^{-/-}$ and mice[86].

It is known that microbiota aids in the brain development and metabolism[87]. The gut microbiota plays an important role in regulation of the HPA axis, particularly, in modulating the host's response to stress[88]. For example, the gut microbiota initiates signaling which affects neurons involved in motor control and anxiety-like behavior[89] independently of inflammation, GI, or vagal signaling[90]. In another study, GF rats

exhibited decreased social communication, increased anxiety-like behavior and neuroendocrine response to acute stress[91]. Finally, GF mice had exaggerated responses to stress indicated by the elevated concentration of stress hormones ACTH and corticosterone relative to their non-GF genetically similar counterparts. GF mice also expressed decreased levels of brain-derived neurotrophic factor in the cortex and hippocampus, which was reversed by introducing *Bifidobacterium infantis* in the early postnatal stage[92]. Together these studies demonstrate the importance of microbiota in the host response to stress mediated by the HPA axis.

There is further evidence that hyper-responsiveness of the HPA axis can be reversed by probiotic bacterial species. In mice, supplementation with *Lactobacillus rhamnosus* (*JB*-1) has been shown to regulate the expression of the main inhibitory neurotransmitter CNS γ-aminobutyric acid (GABA)[93]. Furthermore, supplementation with *L. rhamnosus* decreased plasma concentrations of corticosterone and resulted in reduced anxiety and depression-like behavior in mice[93]. Another probiotic, *Bifidobacterium longum* NCC3001, normalized anxiety-like behavior when given to mice with chronic colitis[90]. Additionally, administration of a probiotic formulation consisting of *Lactobacillus helveticus* R0052 and *B. longum* R0175 for 30 days decreased anxiety-like behavior in rats and alleviated physiological distress and urinary cortisol in healthy humans[94].

It has been determined that exposure to stressors, including social stress and response to environmental stressors, changes composition and density of the gut microbial community[95]. For instance, social stress decreased the genus *Bacteroides* and

increased the abundance of the genus *Clostridium* in mice. Stress induced an increase of serum concentrations of cytokines IL-6 and MCP-1, which were inversely correlated with the relative abundance of *Coprococcus*, *Dorea* and *Pseudobutyvibrio* species[96]. Interestingly, mice pre-treated with antimicrobials to reduce microbiota did not develop increases in IL-6 and MCP-1, suggesting that the observed increased cytokines in stressed mice was induced by microbiota[96]. Research has shown that stress in early life, such as maternal separation, alters behavior, immunity and microbiota in rats. Stressed pups exhibited increased systemic concentrations of plasma corticosterone as well as TNF-α and INF-γ along with increased visceral sensation[97]. Administration of probiotics ameliorated the signs of gut dysfunction and decreased corticosterone concentrations in these rat pups[97b]. The probiotic *Lactobacillus farciminis* was found to be effective in alleviating intestinal permeability as well as corticosterone, ACTH and pro-inflammatory cytokine concentrations induced by LPS treatment in female rats[98].

Temperament in animals is associated with responsiveness of the HPA and resting levels of cortisol[99] and is associated with differential immune response to bacterial infection[100]. In mice, gut microbiota may regulate HPA axis and stress responsiveness, possibly through directly regulating neuronal expansion and morphology. For example, microbiota-deficient mice exhibited expansion of amygdala and hippocampus[101]. Interestingly, in young children an association has been discovered between temperament and the microbiome[102]. Particularly, increased surgency/extraversion, sociability and high-intensity pleasure was associated with

increased alpha diversity in both sexes[102]. In finishing cattle calm temperament was associated with increased *E. coli* O157:H7 shedding following transportation stress[99a].

1.5.2. Stress and microbiome: role in S. *Typhimurium pathogenesis*

S. Typhimurium induces self-limiting enteritis characterized by neutrophil infiltration, submucosal edema, goblet cell depletion, epithelial disruption and crypt abscesses[1]. In the gut, *Salmonella* employs strategies that allow it to compete with resident microbiota and eventually overcome colonization resistance.

1.5.2.1. Dysbiosis benefits *S.* Typhimurium

Disturbance in microbiota density and composition have long been associated with increased susceptibility to salmonellosis. Mice infected with *S.* Typhimurium suffer systemic infection but about 95% do not develop gastrointestinal illness characteristic of *S.* Typhimurium infection in humans. Treatment of mice with a single dose of streptomycin 24 h prior to intragastric inoculation with *S.* Typhimurium leads to dramatically reduced microbiota density and diversity. Consequently, transient disruption of colonization resistance allows *S.* Typhimurium to establish itself in the cecum and colon as fast as 8-12 h post-infection, and to induce acute mucosal inflammation in mice. These observations have led to use of the streptomycin-treated mouse as a model of *Salmonella*-induced diarrhea in humans[103]. There is a strong correlation between an antibiotic-induced (streptomycin, vancomycin and metronidazole) microbiota shift and subsequent severity of *S.* Typhimurium–induced infection[104]. Antibiotic treatment leads to a steep decrease in total bacterial microbiota numbers and is associated with increased systemic translocation of *S.* Typhimurium and

prolonged intestinal inflammation relative to untreated mice infected with *S.* Typhimurium[103a] (Fig. 4).

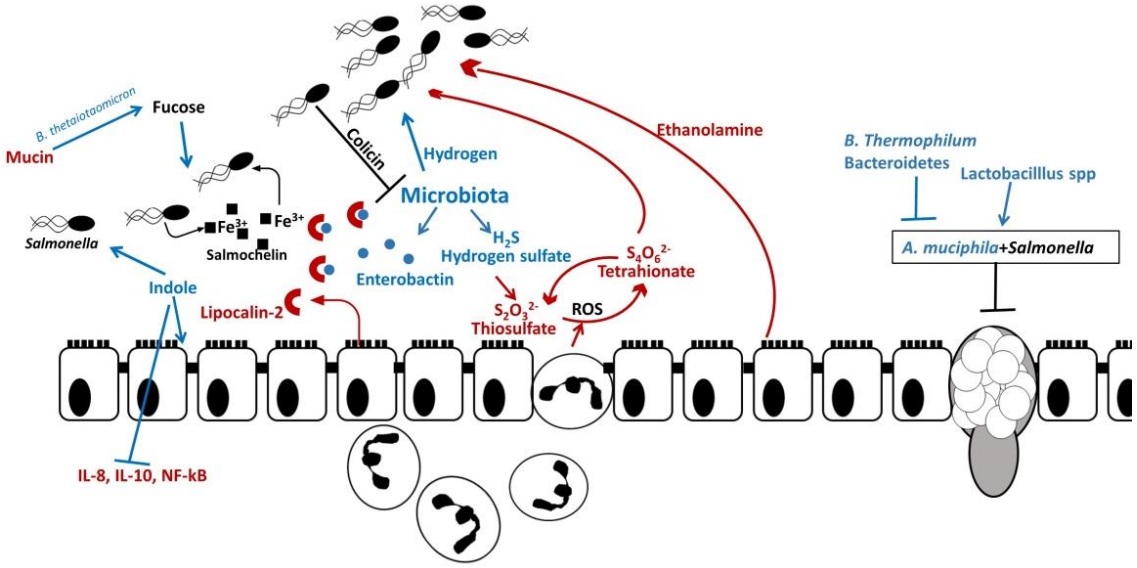

Figure 4: *Salmonella* outcompetes resident microbiota in the gut. Host and resident microbiota protects against *Salmonella* colonization: indole secreted by commensals promotes intestinal health by decreasing secretion of proinflammatory cytokines and by decreasing Salmonella virulence gene expression and certain bacteria (*B. thermophilum* and *Bacteroidetes*) are associated with increased resistance to *Salmonella*. *Salmonella* uses microbiota-produced hydrogen for initial establishment in the intestine. *Salmonella* induces secretion of ROS and antimicrobial peptides by neutrophils and epithelial cells. While *Salmonella* is resistant to these antimicrobial factors, they are harmful to commensal microbes. In the inflamed intestine, microbiota-produced hydrogen sulfate is converted by the host to tetrathionate, which is used by *Salmonella* as an electron acceptor for anaerobic respiration and allows *Salmonella* to utilize ethanolamine, produced by the host as carbon source. Additionally, *Salmonella* – produced bacteriocin, colicin, inhibits resident *E. coli* in the intestine. Thus *Salmonella*-induced inflammation leads to dysbiosis and allows *Salmonella* to propagate in the gut. Some resident bacterial species contribute to *Salmonella* infection. For example, *A. muciphila* inhibits mucin production and aids *Salmonella* in decreasing goblet cell population. Additionally, fucose, which is cleaved from mucin by *B. thetathiomicron* is utilized by *Salmonella* at the initial colonization stage[8].

Intestinal inflammation helps WT and avirulent *Salmonella* to compete with microbiota and enhances *Salmonella* colonization in mice. However, the avirulent strain, which lacked two virulence-associated type III secretion systems, but not WT *Salmonella* was outcompeted by microbiota in the streptomycin mouse model. Additionally, WT was able to alter the microbiota composition (*Salmonella* was the predominant species, ~90%) in streptomycin-treated mice compared to the avirulent strain[105].

1.5.2.2. *S.* Typhimurium induces dysbiosis

S. Typhimurium is able to modify gut microbiota even without antimicrobial pre-treatment. For example, oral *S.* Typhimurium infection induces SPI-1-dependent dysbiosis which is reversed within a month after inoculation[106] (Fig. 4). Interestingly, a change in microbiota composition preceded *S.* Typhimurium-induced diarrhea, suggesting a host-pathogen interaction resulting in dysbiosis but unrelated to diarrhea[106]. One of the drivers of *Salmonella*-induced dysbiosis is the depletion of Clostridia, which are the main butyrate producers. Decrease of cecal butyrate leads to elevated oxygenation of colonocytes and increased aerobic proliferation of Bacilli, Bacteroidia and Gammaproteobacteria (including *S.* Typhimurium)[107]. Additionally, mice with low complexity microbiota (8-40 bacterial strains) were susceptible to *S.* Typhimurium-induced colitis without need for antibiotic treatment and failed to clear pathogen from the gut lumen. Clearance of *Salmonella* can be facilitated by transferring microbiota from a conventional mouse to mic with the low complexity microbiota [108]. Interestingly, mice colonized with commensal *E. coli* were more susceptible to *Salmonella*-induced

inflammation, suggesting that closely related phylotypes displayed correlated abundances and pathogen blooms[108a]. These differences are not limited to mammalian hosts. In newly hatched chicks, *S.* Typhimurium does not cause clinical disease; however, it induces minor changes in the cecal microbiota[109]. In adult chickens, *S.* Typhimurium infection led to an increase in *Enterobacteriaceae* and decrease in *Ruminococceae* in the cecum[110] although, overall changes in microbiota composition were much less pronounced compared to mammals.

Microbial metabolites can be beneficial for the initial pathogen assimilation in the gut. It has been shown that murine cecal microbiota produce approximately 2 µM hydrogen. Moreover, the genes encoding enzymes involved in the H_2 production are abundant within the gut microbiota. In *S.* Typhimurium, H_2 –consuming hydrogenases (*hyd*) are required for initial establishment in the intestine[45, 111] and overcoming colonization resistance in SPF mice. Whereas in the GF mouse the ability to consume H_2 is not required[111b]. Interestingly, the inability of the *hyd3* mutant to consume microbiota-derived H_2 did not affect *S.* Typhimurium colonization of systemic sites in the mouse[111a]. Therefore, H_2-utilization is considered to be a general feature of *S.* Typhimurium necessary for gut colonization.

1.5.2.3. *S.* Typhimurium exploits intestinal inflammation to compete with microbiota

Intestinal inflammation provides metabolic advantages to *Salmonella* to compete with resident microorganisms. Under normal conditions, the mammalian gut converts harmful hydrogen sulfide to thiosulfate[112]. During inflammation induced by *S.*

Typhimurium virulence factors SPI-1 and SPI-2, thiosulfate is oxidized to tetrathionate by reactive oxygen species. Subsequently, *S.* Typhimurium utilizes tetrathionate as an electron acceptor for anaerobic respiration, which allows the pathogen to outcompete the microbiota in the inflamed gut[6d]. Tetrathionate also acts as an electron acceptor allowing *S.* Typhimurium to utilize ethanolamine released from the host tissue. The *ttrA* gene encoding tetrathionate reductase subunit A is essential for *S.* Typhimurium growth in ethanolamine *in vivo* while the majority of gut microbiota are unable to use ethanolamine for fermentation[113].

S. Typhimurium employs efficient mechanisms to compete for nutritional iron in the inflamed intestine. One of the mechanisms the pathogen uses is an induction of lipocalin-2 production by neutrophils. Lipocalin-2 is an antimicrobial protein which prevents iron acquisition in the intestine by commensal bacteria by binding to the siderophore, enterobactin. *Salmonella* Typhimurium produces a glycosylated variant of enterobactin, salmochelin, which utilizes transporter IroN. Salmochelin is not bound by lipocalin-2 and, therefore, gives *Salmonella* an advantage over IroN-negative bacteria[114]. The lipocalin-2 resistance system has also been found in non-pathogenic Nissle strain of *E. coli* which is able to outcompete *S.* Typhimurium in a mixed infection and reduce pathogen numbers, making it a promising probiotic for preventative and therapeutic use[115]. *S.* Typhimurium also utilizes ferrous iron as co-repressor of siderophore synthesis in order to avoid damage by ROS secreted by immune cells. Production of iron-depleting agents such as lipocalin-2, lactoferrin, ROS and RNS by macrophages triggers ferric uptake regulator (*fur*)-dependent responses which control defense against

peroxide. In addition to defensive mechanisms *S.* Typhimurium employs offensive tactics to compete in the gut such as production of antimicrobial bacteriocins. Intestinal inflammation triggers pore-forming, *fur*-dependent bacteriocin, colicin lb, which gives *Salmonella* an advantage over colicin-sensitive *E. coli* strains and allows *Salmonella* to "bloom" in the inflamed gut[116]. In addition to inducing intestinal blooms of the pathogen, intestinal inflammation combined with microbiota disturbance has been shown to increase genetic material exchange between *Salmonella* and *E. coli* which could be prevented by commensal microbiota[117].

1.5.2.4. Microbiota regulates susceptibility to infection

Some commensal bacteria develop synergistic relationships with *S.* Typhimurium. One such example is *Akkermansia muciphila* which causes increased pathology during *S.* Typhimurium infection by disturbing mucus production and sulfation. A combination of *A. muciphila* and *S.* Typhimurium significantly reduced numbers of mucin-filled goblet cells in the murine intestine, compared to uninfected mice or mice inoculated with either *A. muciphila* or *S.* Typhimurium alone. Presence of both bacteria increased *S.* Typhimurium proportions to 94% from 2.2% in an *S.* Typhimurium mono-infection. Additionally, expression levels of inflammatory cytokines INF-γ, IP-10, TNF-α, IL-12, IL-6 and IL-17 were significantly increased in mice infected with both bacteria compared to a single infection or uninfected mice. Thus, during *S.* Typhimurium infection commensal *A. muciphila* turns into a pathobiont and contributes to disease symptoms[118].

Additionally, the gut microbiota induces systemic IgG production in response to GI infection, protecting the host from *Salmonella*-induced bacteremia. IgG production is accomplished in large part through TLR4 signaling in response to gram-negative murein lipoprotein[119]. Particular members of the resident microbial community have been associated with increased resistance to infection. A study performed in conventional streptomycin-treated mice has shown that the Bacteroidetes phylum was associated with increased resistance to *S.* Typhimurium-induced colitis while *Lactobacillus* sp. was associated with decreased resistance[104]. Also, a combination of commensal microbiota, particularly *Clostridium cironiae*, and gut metabolites exhibited antibacterial activity towards *S.* Typhimurium as well as reduced cell invasion[120]. Finally, supplementation with the probiotic *Bifidobacterium thermophilum* RBL67 (RBL67) in combination with prebiotics (fructo- (FOS), galacto- (GOS) and mannan- (MOS) oligosaccharides) decreased *S.* Typhimurium colonization in a swine proximal colon simulation model[121]. Co-culture of *B. thermophilum* RBL67 and *S.* Typhimurium at mid-exponential growth phase resulted in repression of regulatory gene *phoP* and flagella, although expression of SPI-1 genes, SPI-2 genes, and fimbriae was increased. The premature induction of virulence gene expression and reduced motility in *S*. Typhiumurium induced by *B. thermophilum* RBL67 resulted in restricted colonization by the pathogen[122].

1.6. Stress can modify the interaction between host, microbiota and *Salmonella*

Exposure to stress hormones has been shown to increase survival and virulence of *Salmonella* (by the mechanisms described above). For example, exposure of *S.* Typhi to NE and Epi induced release of toxin hemolysin E, which was attenuated by the β

blocker propranolo[123]. In another study treatment of intestinal tissues with 10 µM NE improved the uptake of *S.* Choleraesuis into porcine jejunal Peyer's patches[124]. Likewise, incubation of *S.* Typhimurium in serum-SAPI with 2 mM NE prior to inoculation increased bacterial survival in the swine stomach and bacterial loads in the small intestine, large intestine and feces compared to bacteria grown in LB without NE[125]. It was determined that density of the bacterial inoculum should be low for efficient NE-induced bacterial growth[126]. Additionally, CAs can alter cellular uptake of *Salmonella* at the early infection stages. For example, increased dopamine concentrations at the contra-luminal aspect of the intestine resulted in decreased bacterial recovery from Payer's patches *ex vivo*[127] whereas cortisol increased intracellular *S.* Typhimurium proliferation in primary porcine alveolar macrophage cultures and in mice[128]. The above mentioned effect is attributed to the cortisol-induced activation of regulator protein ScsA in *S.* Typhimurium which leads to increased SCV-production by macrophages allowing increased bacterial proliferation[129].

Not only does stress aid the virulence of the pathogen, but it also increases host susceptibility to *S.* Typhimurium infection. To illustrate, feed withdrawal and heat stress in chickens were associated with increased *S.* Typhimurium numbers and increased intestinal pathology[130]. Treatment of pigs with NE or the NE precursor, 6-hydroxydopamine (6-OHDA), which mimics acute stress, increased *Salmonella* shedding in pigs[131]. Additionally, decreased fecal shedding exhibited by the *qseC* mutant was reversed by treatment of pigs with 6-OHDA prior to infection[131] which suggests that activation of QseC-dependent signaling by NE may not be required for *S.* Typhimurium

virulence *in vivo,* as long there is an increase in stress hormone levels in the host. Another study demonstrated that, 24 h feed withdrawal or dexamethasone injections increased serum cortisol concentrations and *Salmonella* shedding in pigs[128].

Finally, a combination of stress and bacterial infection can affect resident microbiota in the GI tract[132] as well as gut-brain communication (Fig. 5). For example, acute stress combined with *C. rodentium* infection induced memory dysfunction in mice, while infection alone did not result in memory impairment. Daily treatment with probiotics (*L. rhamnosus* (R0011) and *L. helveticus* (R0052)) restored microbiota and prevented memory dysfunction in infected stressed mice. Probiotics also decreased serum cortisol and pro-inflammatory cytokine concentrations in these animals indicating that beneficial bacteria may ameliorate gastrointestinal illness and prevent memory alteration[133]. It is unclear whether the exposure to stress during infection with gastrointestinal pathogens such as *S.* Typhimurium will affect memory or other brain functions in animals and additional study is warranted.

1.7. Concluding remarks and future perspectives

The evidence supports the hypothesis that microbiota communicates with the CNS and ENS through neural, endocrine, immune and humoral links[134] resulting in the microbiota having numerous effects on the health of the host[135]. The gut microbiome helps shape the innate and adaptive immune systems and plays a role in host nutrition and neuroendocrine pathways[136]. Further, mounting evidence suggests that the gastrointestinal microbiome is involved in the development and function of the mammalian nervous system[137 138].

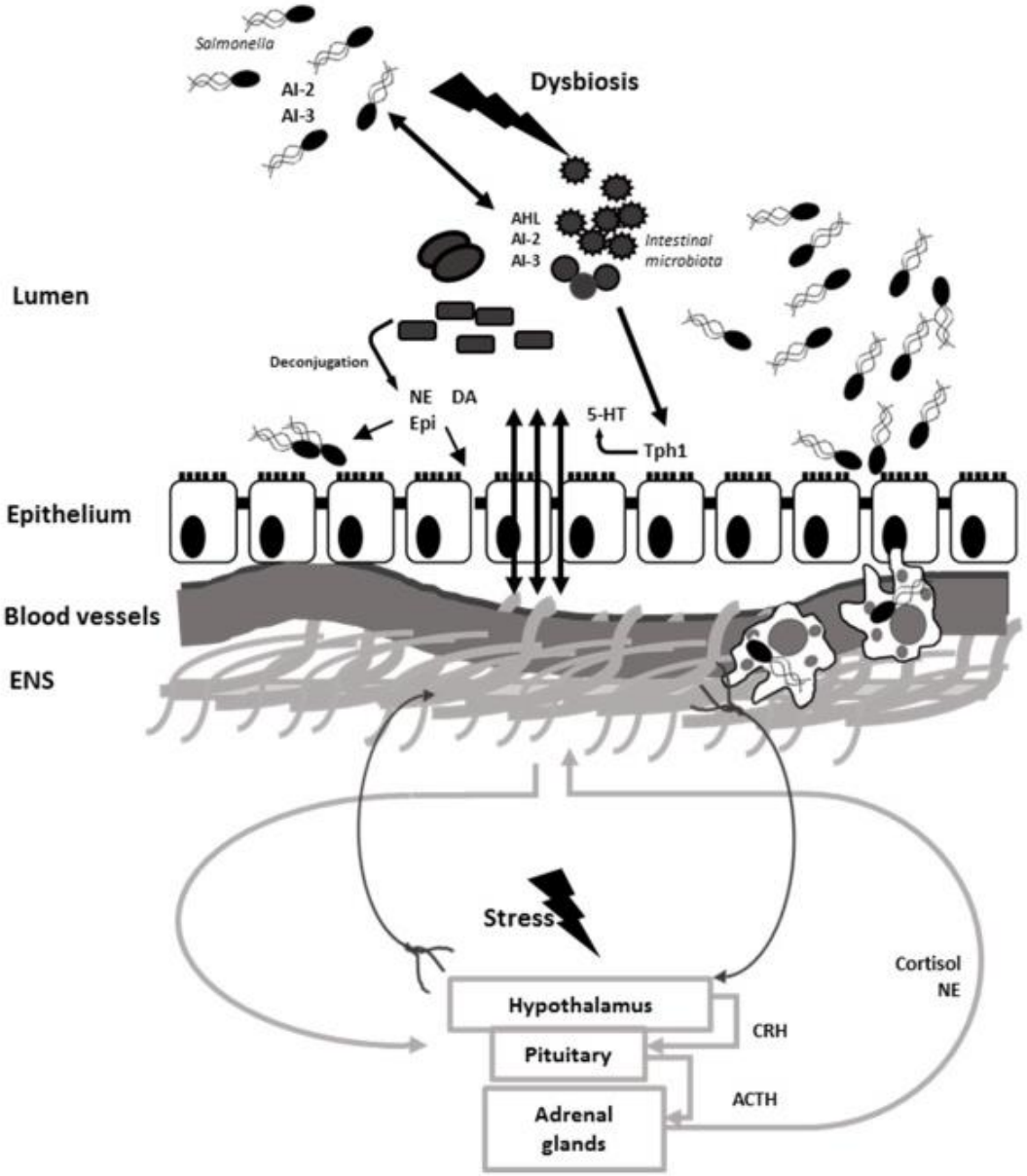

Figure 5: *Salmonella* induces dysbiosis and interferes with gut-brain communication. Neural signals travel via efferent neurons from CNS to the ENS and back via afferent neurons. Microbiota stimulate synthesis and modulates bioavailability of neurochemicals such as 5-HT, NE, DA and Epi which are utilized by the host. Stress stimulates the activation of HPA axis and release of cortisol and catecholamines. NE is released in the gut via spillover from sympathetic neurons. Resident microbiota use an array of quorum sensing signals which can be intercept by *Salmonella*. Catecholamines along with AI-2 and AI-3 QS molecules stimulate *Salmonella* growth and virulence in the intestine. Stress aids to *Salmonella* pathogenesis and induces dysbiosis, which helps *Salmonella* establishment in the intestine and dissemination via phagocytic cells[8].

The enteric pathogen *S. Typhimurium* employs a multitude of tactics to overcome colonization resistance by microbiota including 1) inducing inflammation which modulates nutrient availability by suppressing other bacteria; 2) through complex gene regulation pathways that link quorum signaling and metabolism; and 3) intercepting signals produced by the microbiota and host.

Future research should investigate the multidirectional signaling in the GI tract to aid in our understanding of the communication between the host, resident microbiota and GI pathogens within the challenging environment of gastrointestinal tract.

2. INTEGRATION OF CARBON METABOLISM AND QUORUM SENSING THROUGH THE BARA/SIRA-CSRA AND *LSR* REGULATORY SYSTEMS IN *SALMONELLA* TYPHIMURIUM

2.1. Summary

Autoinducer-2 (AI-2) is a quorum signal that regulates virulence in *Salmonella enterica* Serotype Typhimurium. In addition to AI-2 regulation, LsrR, encoded on the *lsr* operon, suppresses the expression of *Salmonella* pathogenicity island 1 (SPI-1) and flagella-encoding genes. The global regulatory RNA-binding protein CsrA also regulates the SPI-1 Type 3 Secretion System (T3SS) and is, in turn, regulated through the SirA/BarA-CsrBC regulatory cascade. The interaction between the SirA/BarA two-component system and the *lsr* operon is unknown. The goal of this study was to determine whether the SirA/BarA-CsrA regulatory cascade regulates AI-2 quorum sensing (QS) in *Salmonella* Typhimurium. In this study we demonstrate that CsrA directly regulates *lsr* translation by binding to the mRNA of *lsrR* and *lsrACDBFGE* promoters thereby repressing the translation of corresponding genes. We also determined that the expression of *lsrR* is positively regulated by SirA/BarA and CsrBC, which results in an increase in extracellular AI-2 activity. This is counteracted by CsrA binding to the *lsrR* transcript and relieving the expression of AI-2 transporter. Our results demonstrate cross-talk between the SirA/BarA-CsrA and *lsr* regulatory systems in regulating AI-2 QS in *S.* Typhimurium.

2.2. Introduction

Autoinducer-2 (AI-2) quorum sensing (QS) in *S.* Typhimurium is repressed by the transcriptional repressor protein LsrR [139]. During early-log and mid-log growth phase S-ribosylhomocysteinase (LuxS) converts S-adenosylhomocysteine to 4,5 dihydroxy-2,3-pentanedione (DPD) which is cyclized into (2R, 4S)-2-methyl-2,3,3,4-tetrahydroxytetra-hydrofuran (AI-2)[140] and is secreted into the extracellular space via an unknown mechanism. Extracellular AI-2 is actively transported into the bacteria by the *lsrACDB*-encoded ABC transporter[139]. Following intracellular phosphorylation by LsrK[139], AI-2 prevents binding of LsrR to *lsr*, allowing the transcription of *lsrACDBGF*- and *Salmonella* pathogenicity Island 1 (SPI-1) – encoded genes[139, 141]. In the cell, AI-2 is converted to dihydroxyacetone phosphate and acetyl-CoA by LsrF[142] and LsrG[139] and extracellular AI-2 is virtually non-detectable by early stationary phase growth[17].

In *Salmonella*, LuxS and AI-2 regulate the expression of genes required for motility, biofilm formation, virulence, translation, transcription and other key cellular functions[22]. LuxS induces swimming and swarming motility as well as negatively regulates expression of virulence-related genes *hilA* and *hilD*[22]. LsrR-mediated QS negatively regulates SPI-1 and flagella-encoding genes, thus regulating *S.* Typhimurium invasion into mammalian epithelial cells[24].

Regulation of the SPI-1 T3SS is complex and requires the coordination of gene expression with response to environmental signals such as osmolarity, pH, and oxygen tension. The global regulatory RNA-binding protein CsrA regulates the SPI-1 T3SS and is, in turn, regulated through the SirA/BarA-CsrBC-CsrA regulatory cascade. The

SirA/BarA two-component system positively regulates the expression of SPI-1[143], SPI-2[63b] and SPI-4 genes[144]. SirA controls the expression of SPI-1 and SPI-2 genes by regulating HilD expression, and downstream *hilA* and *ssrAB* expression via CsrBC-CsrA[63b, 143d, 145]. SirA binds to the *csrB* and *csrC* regulatory regions and activates these small regulatory RNAs[63b]. CsrB and CsrC sequester CsrA, which allows HilD expression[63b]. CsrA also binds multiple targets within SPI-1 and virulence genes in other pathogenicity islands and within the core genome[146]. It has been shown that deletion of *barA* has little effect on *Salmonella* virulence in BALB/c mice, while deletion of *sirA* reduced virulence associated with decreased expression of *hilA, invF* and *sipC*[147].

In a study measuring *Salmonella* gene expression in in bovine ileal loops, *lsrD* expression was increased at 15, 30, 60, 120, 240 and 480 min post-inoculation (Lawhon et al, unpublished data). This suggests that the *lsr* operon encoded AI-2 transporter may be important for *S.* Typhimurium virulence *in vivo*. Additionally, microarrays performed on WT and *csrA* strains of *S.* Typhimurium have shown that *lsrBFGE* were downregulated whereas *lsrR* was slightly (1.5 fold) upregulated in a *csrA* mutant *in vitro*[148] indicating that there may be a cross-talk between SirA/BarA-CsrA and *lsr* regulatory systems.

To date, LsrR is the only known transcriptional regulator of AI-2 in *Salmonella*. It is unknown whether SirA/BarA and CsrA regulatory systems are involved in the regulation of the *lsr* operon and AI-2 QS in S. Typhimurium. Here we show that the

SirA/BarA-CsrA regulatory cascade is involved in the regulation of *lsr* and, subsequently, AI-2 QS in *S.* Typhimurium.

2.3. Results

2.3.1. SirA/BarA-CsrA regulatory system regulates lsrR *and* lsrD *expression and AI-2 in* S. *Typhimurium*

In *S.* Typhimurium SirA can be phosphorylated by BarA or by acetyl-phosphate[149]. The products of *ackA* and *pta* genes convert acetate and acetyl-Co-A respectively to acetyl phosphate which can phosphorylate SirA independent of BarA[143c, 147]. The DNA-binding response-regulator SirA stimulates the expression of small regulatory RNAs CsrB and CsrC by binding to the regulatory regions of *csrB* and *csrC* thus inducing *csrBC* translation[63b, 143d, 148]. LsrD is a part of the *lsr*-encoded AI-2 transporter, whereas LsrR suppresses the *lsr* operon and therefore suppresses AI-2 internalization. To investigate whether the SirA/BarA-CsrA regulatory cascade regulates the expression of *lsrR* and *lsrD*, we analyzed the expression of transcriptional fusions of the *lsrR* and *lsrD* genes to the *lacZ* reporter gene in *S.* Typhimurium. Bacterial strains were grown in LB medium to reach mid-exponential growth phase (OD_{600} = 0.6-0.7) which corresponds to the beginning of active AI-2 internalization in WT of *S.* Typhimurium. At the mid-exponential growth phase, expression of *lsrR-lacZ* was decreased in a *sirA* mutant, suggesting that SirA is involved in *lsrR* regulation (Fig. 6B). Additionally, in the absence of *csrBC* the *lsrR-lacZ* and *lsrD-lacZ* expression was decreased at mid-exponential growth phase (Fig. 6B and Fig. 7B), suggesting that regulatory RNAs CsrB and CsrC positively regulate *lsrR* and *lsrD* expression.

Transcriptional activity of *lsrR-lacZ* and *lsrD-lacZ* was increased in the *csrA* mutant compared to that of wild-type (WT) (Fig. 6B and Fig. 7B). These results indicate that CsrA suppresses expression of *lsrR* and *lsrD* at the mid-exponential growth phase in *S.* Typhimurium.

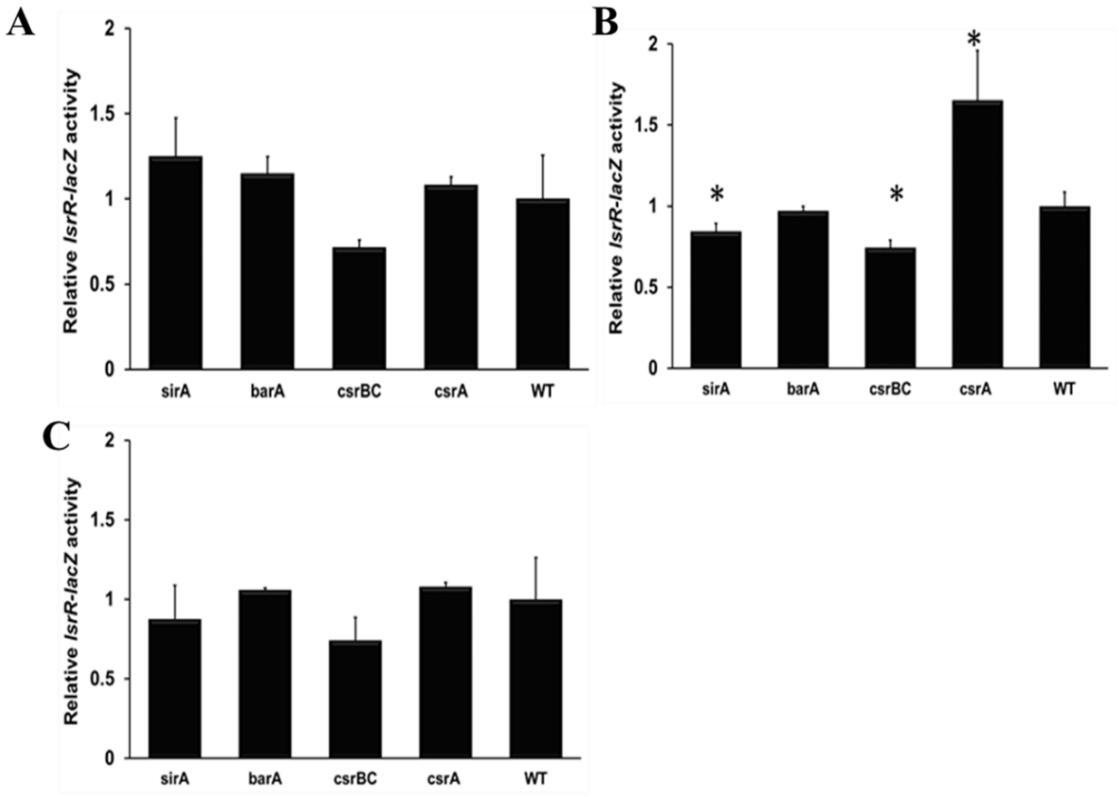

Figure 6: SirA/BarA-CsrA regulatory cascade regulates *lsrR* expression. *β*-galactosidase activity of the *lsrR-lacZ* was determined in the following strains: WT, *sirA*, *barA*, *csrBC* and *csrA*. *lsrR-lacZ* activity was measured at (A) early-exponential, (B) mid-exponential and (C) late-exponential growth phase. Bars represent standard deviation. *$P < 0.05$ when compared to WT.

During early-exponential growth, *lsrD-lacZ* expression was increased in the *csrA* mutant compared to WT (Fig. 7A). These data suggest that initially CsrA represses *lsrD* while at later stages of growth CsrA represses both *lsrR* and *lsrD*. At the late-

exponential growth phase *lsrD* is repressed by CsrA as *lsrD-lacZ* expression is increased in *csrA* mutant (Fig. 7C). Expression of *lsrR-lacZ* was not different in the *csrA* mutant as compared to the WT at the early-and late-exponential growth phase (Fig. 6A and Fig. 6C). These results are unexpected as they suggest that CsrA suppresses both *lsrR* and *lsrACDBFG* simultaneously during the mid-exponential growth phase. The only known function of *lsrACDBFG* is associated with AI-2 transport and degradation in the cell [139, 141b]. We suggest that when bacteria are present in low numbers, *lsrACDBFG* is suppressed by CsrA and LsrR. CsrA also suppresses *lsrACDBFG* during the mid-exponential growth phase allowing AI-2 internalization. Internalized AI-2 is phosphorylated thereby inhibiting LsrR binding to the *lsr* promoter and allowing further AI-2 internalization[20]. During the late exponential growth phase, AI-2 is no longer produced and internalized so *lsrACDBFG* is suppressed.

To determine whether the SirA/BarA-CsrA system regulates extracellular AI-2 concentration in *S.* Typhimurium, we measured extracellular AI-2 activity in cell-free culture media at various stages of growth. AI-2 activity was measured using a reporter system which measure bioluminescence in *Vibrio harveyi* in response to AI-2. At the mid-exponential phase of growth, extracellular AI-2 activity was higher in WT *S.* Typhimurium compared to *sirA*, *barA*, and *csrBC* mutants (Fig. 8A).

Extracellular AI-2 activity did not differ between the *csrA* mutant and WT at the mid-exponential phase of growth (Fig. 8B) but was significantly different at the late-exponential growth phase (Fig. 8C). At the late-exponential growth phase, extracellular AI-2 activity was almost non-detectable in media from WT cultures but in media from

cultures of a *csrA* mutant, extracellular AI-2 activity was high (Fig. 8C). These results indicate that SirA/BarA-CsrA regulatory cascade impacts AI-2 activity in *S.* Typhimurium. This suggests that the SirA/BarA-CsrA regulatory pathway regulates transcription of the *lsr* operon and subsequent change in AI-2 uptake, where SirA/BarA-CsrBC induces *lsr* transcription and AI-2 uptake whereas CsrA suppresses these activities.

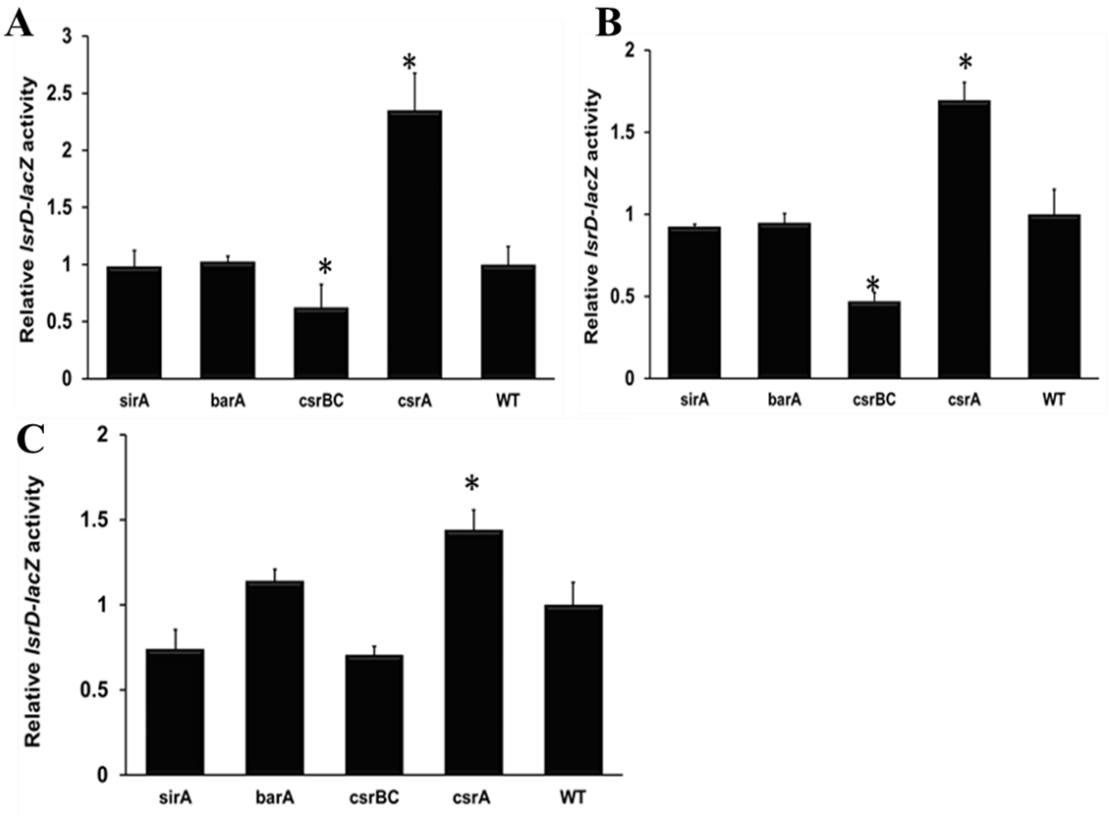

Figure 7: CsrA regulates *lsrD-lacZ* expression at various stages of growth. *β*-galactosidase activity of the *lsrD-lacZ* was determined in the following strains: WT, *sirA*, *barA*, *csrBC* and *csrA*. *lsrD-lacZ* activity was measured at (A) early-exponential, (B) mid-exponential and (C) late-exponential growth phase. Bars represent standard deviation. *$P < 0.05$ when compared to WT.

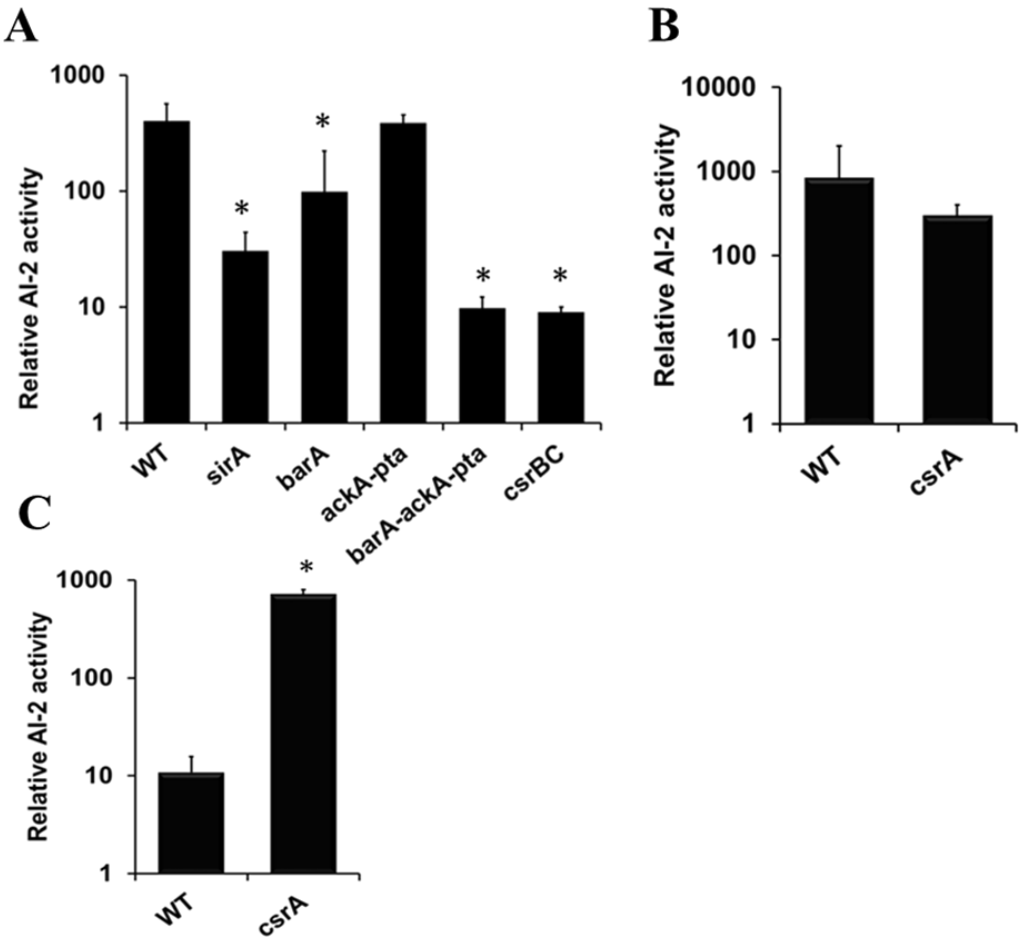

Figure 8: SirA/BarA-CsrBC regulatory cascade and CsrA have an opposite effect on extracellular AI-2 activity. Bars represent standard deviation. *$P < 0.05$ when compared to WT. (A). Extracellular AI-2 activity in cell-free culture fluids was determined at OD_{600} = 0.6-0.7 in the following strains: WT, *sirA*, *barA*, *ackA-pta*, *barA-ackA-pta*, *csrBC* and (B) WT and *csrA*. (C). Extracellular AI-2 activity in cell-free culture fluids was determined at OD_{600} = 0.9-1.0 in the following strains: WT and *csrA*.

2.3.2. CsrA binds to the lsr *promoter*

CsrA binds to sequences with homology to the Shine-Dalgarno sequence within the regulatory region of genes[63b, 150]. Potential binding sites in *lsr* 5' untranslated region were determined by mfold web server (Fig. 9)[151].

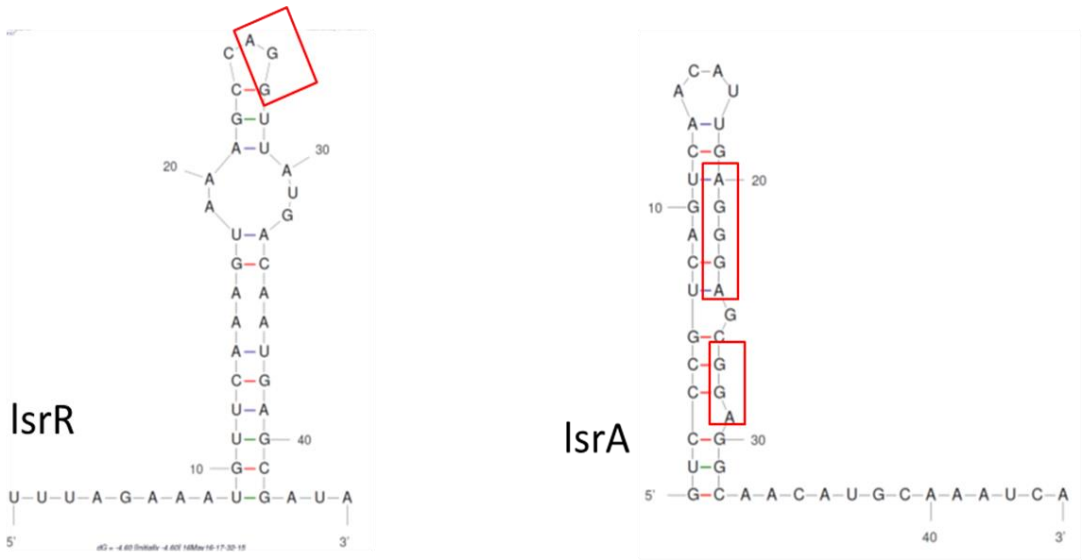

Figure 9: Predicted folding of *lsrR* and *lsrA* oligonucleotides. Potential CsrA-binding regions are in red box.

To determine whether CsrA binds to the *lsrR* and *lsrACDBFGE* promoters, we performed differential radial capillary action of ligand assay (DRaCALA). Incubation of 500 pM of *lsrR* mRNA or *lsrA* mRNA with 12.5, 25, 50, 100, 200, 40, and 800 nM of CsrA resulted in the concentration-dependent binding of both transcripts (Fig. 10A and 10B). These results indicate that CsrA binds to the promoters of *lsrR* and *lsrACDBFGE*, thus directly repressing *lsrR* and *lsrD* translation.

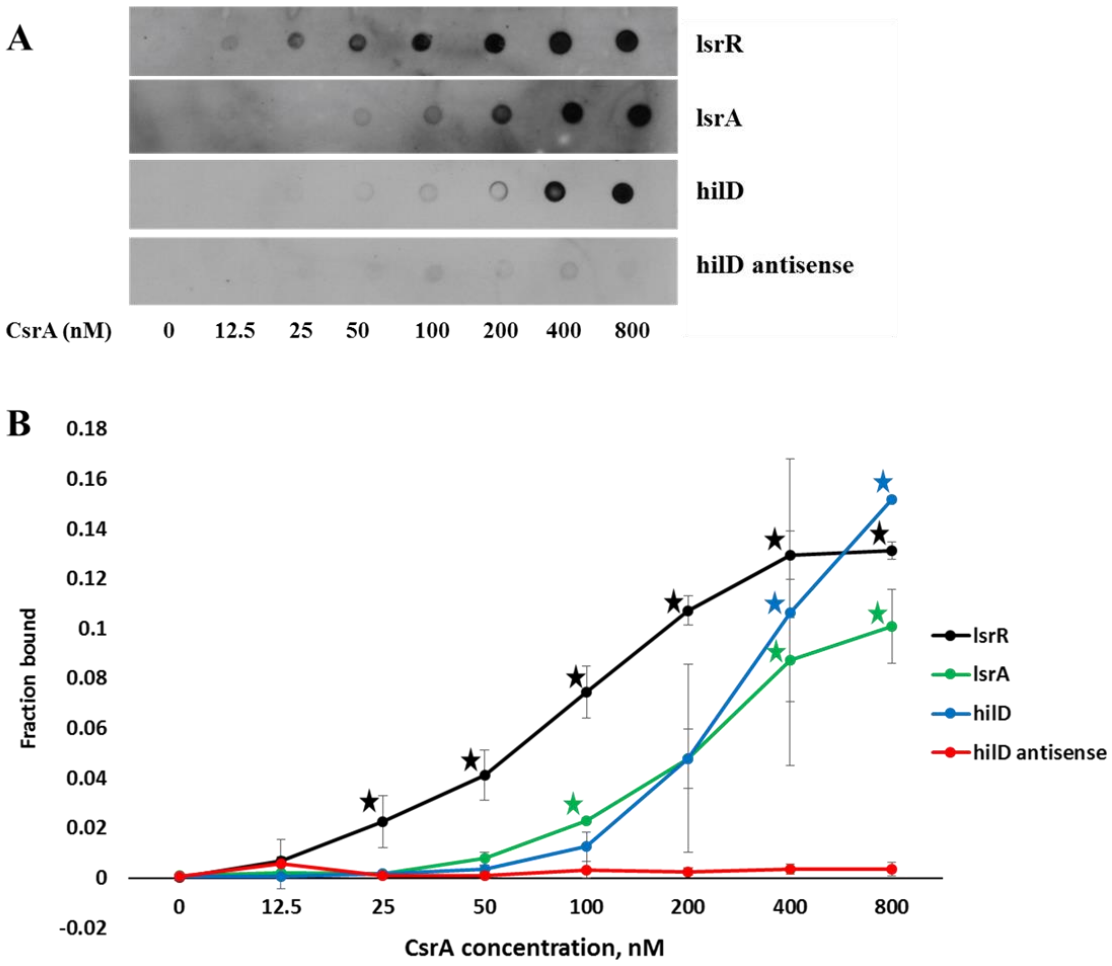

Figure 10: CsrA interaction with *lsrR* and *lsrACDBFGE* RNA.
5'-end labelled RNA (500 pM) of each gene was incubated in the presence (+) and absence (-) of increasing concentration of CsrA. (A) DRaCALA indicates that CsrA binds to *lsrA, lsrR, hilD* but not to *hilD* antisense RNA. (B) Determination of the relative binding activity of CsrA to *lsrR, lsrA, hilD* and *hilD* antisense RNA. Bars represent standard deviation, *$P < 0.05$ when compared to *hilD* antisense.

2.3.3. SirA/BarA-LsrR regulatory systems regulate biofilm formation in S. *Typhimurium*

BarA/SirA, CsrBC, CsrA and LsrR regulate multiple virulence factors. Previous work demonstrated that biofilm formation in *S.* Typhimurium *sirA*, *barA* and *csrBC* mutants was decreased relative to wild type[152]. Additionally, AI-2 has been shown to

regulate biofilm formation in *S.* Typhimurium where biofilm formation was decreased in an AI-2 deficient *luxS* mutant[153]. To evaluate whether the LsrR regulatory system regulates biofilm formation is *S.* Typhimurium, we compared the ability to form biofilms of *lsrD, lsrR, barA-ackA-pta, ackA-pta, barA, sirA, csrB, csrC, csrBC* and *csrA* mutants to that of WT. Strains were grown in LB broth, and biofilm formation was evaluated 36 h post-inoculation. Acetate kinase (*ackA*) and phosphate acetyltransferase (*pta*) convert acetate and acetyl-Co-A respectively to acetyl phosphate. SirA can be phosphorylated by BarA or by acetyl phosphate, therefore lack of all three genes *barA, ackA,* and *pta* results in loss of phosphorylation of SirA[147, 154].

Mutants in *lsrR, lsrD, barA-ackA-pta, barA, sirA, csrB, csrBC* and *csrA* had decreased biofilm formation compared to WT, whereas an *ackA-pta* mutant had increased biofilm formation when compared to WT (Fig. 11). We confirm previous data [152] that SirA-CsrBC induces biofilm formation in *S.* Typhimurium. Additionally, we determined that *lsr* operon may stimulate biofilm formation. We also determined that acetyl phosphate may be an important factor which limits biofilm formation in *S.* Typhimurium. Contrary to previous study[153] we did not observe decrease in biofilm formation in *luxS* mutant. The *lsrR* and *lsrD* mutants had slight, although significant decreases in biofilm formation.

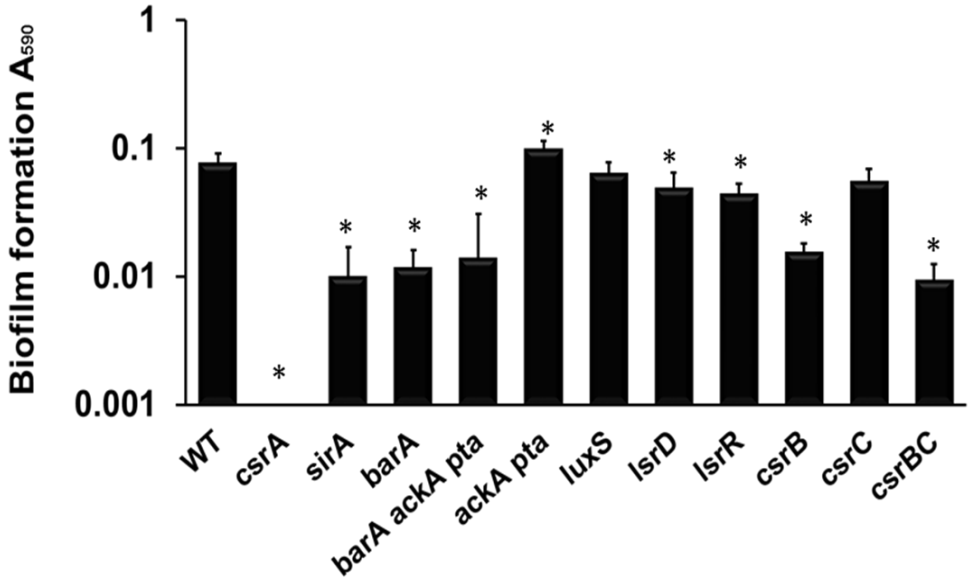

Figure 11: SirA/BarA-LsrR regulatory cascade regulates biofilm formation in *S.* Typhimurium. Biofilm formation was determined in the following strains: WT, *csrA, sirA, barA, barA-ackA-pta, ackA-pta, luxS, lsrD, lsrR, lsrK, csrB, csrC* and *csrBC* after 36 h of incubation at 37°C. Biofilms were stained for 15 min with crystal violet for colorimetric detection.
Bars represent standard deviation. ****P* **< 0.05 when compared to WT.

2.3.4. RbsB as an alternative transporter for AI-2 in S. *Typhimurium*

The ABC transporter encoded by *lsr*ACDB is the only known AI-2 transporter to date[18a]. However, lack of *lsr*ACDB does not completely halt AI-2 internalization[18a]. The microarray study has shown that there may be an alternative AI-2 transporter in S. Typhimurium, encoded by *rbsB* (ABC superfamily (membrane), D-ribose high-affinity transport protein)[22]. In the above study, *rbsB* expression was increased in the absence of *luxS*. Additionally, *rbsB* has been found to regulate AI-2 internalization and virulence in *Haemophilus influenza*[155]. To investigate whether RbsB is an alternative transporter of AI-2 we made *lsrD*, *rbsB* and *rbsB-lsrD* deletion mutants to evaluate extracellular AI-2 concentrations in the absence of one or both transporters. We assessed extracellular AI-2

concentrations in WT, *luxS* (negative control), *lsrD*, *rbsB* and *lsrD-rbsB* strains of *S.* Typhimurium.

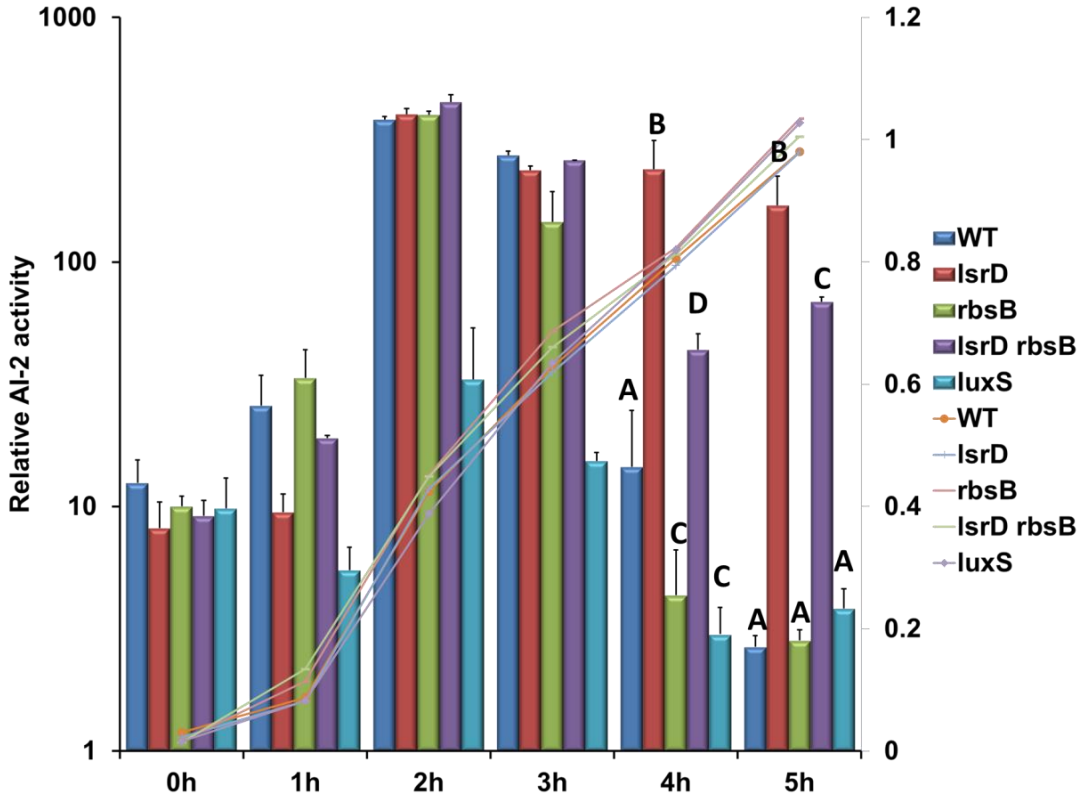

Figure 12: Lack of *rbsB* does not result in decreased AI-2 transport compared to WT at 4h and 5h. (A, B, C, D) indicate *P*< 0.05.

As described before[156], deletion of *luxS* resulted in dramatically decreased extracellular AI-2 (Fig. 12). Deletion of *lsrD* resulted in increased extracellular AI-2 levels compared to WT at 4h and 5h of growth (Fig. 12). However, deletion of *rbsB* did not increase extracellular AI-2 levels compared to WT. Deletion of both *lsrD* and *rbsB* resulted in an intermediate increase of extracellular AI-2 concentration, compared to WT

and *lsrD* (Fig. 12). Therefore we determined that *rbsB* may be involved in AI-2 regulation in *S.* Typhimurium; however, it is an unlikely AI-2 transporter.

2.4. Discussion

Based on previous reports[24, 63b, 148] and information generated in this study we determined that there is a cross-talk between the SirA/BarA-CsrA regulatory cascade and AI-2 QS. First, we determined that at mid-exponential growth phase, extracellular AI-2 in media from cultures of mutants lacking the response regulator, SirA, or the small regulatory RNAs, CsrB and CsrC, was decreased, implying that SirA and CsrBC suppress AI-2 internalization (Fig. 8B). Additionally, SirA and CsrBC positively regulated *lsrR-lacZ* transcription (Fig 6A) indicating that SirA and CsrBC positively regulate LsrR thus suppressing AI-2 internalization in *S.* Typhimurium.

In *S.* Typhimurum CsrA regulates a variety of functions such as growth[157], invasion[157], motility[148], and biofilm formation[152]. A recent study determined the global RNA recognition pattern of CsrA and identified multiple CsrA targets in SPI-1 and SPI-2[146]. Interestingly, this study also determined CsrA binding to *lsrC*[146]. We demonstrated that at mid-exponential growth phase CsrA negatively regulated *lsrR* expression and therefore positively regulates AI-2 internalization (Fig 6A). Next, we demonstrated that CsrA binds directly to the *lsrR* transcript (Fig. 10A) thus preventing translation. This leads to de-repression of *lsrABCD* thereby allowing AI-2 internalization. These data suggest that at the mid-exponential growth phase, SirA/BarA-CsrBC positively regulate *lsrR* expression thus repressing AI-2 internalization whereas CsrA directly suppresses *lsrR* translation and stimulates AI-2 internalization.

Regulation of QS and AI-2 intake are tightly controlled in *S.* Typhimurium because they regulate key virulence factors including flagella and SPI-1[24, 26]. AI-2-deficient *S.* Typhimurium display impaired *invF*-dependent virulence in mice[24]. AI-2 uptake in mid-exponential growth phase ensures that LsrR will be suppressed allowing SPI-1 expression at the late-exponential growth phase. In this study we determined that at the early-exponential growth phase, CsrB and CsrC positively regulate *lsrD* expression whereas CsrA acts as a repressor of *lsrD* expression indicating that CsrBC-CsrA system is involved in the AI-2 internalization during early stages of bacterial growth. We demonstrated that CsrA also binds to the *lsrD* transcript, suggesting that CsrA is a repressor of the *lsr* operon. However, during the early-exponential growth phase CsrA represses *lsrD* expression but not *lsrR* expression. This suggests that initially CsrA represses *lsrD* thus repressing AI-2 internalization and then during the mid- and late-exponential growth phase *lsrD* is still repressed; however, CsrA also suppresses *lsrR* at the mid-exponential growth phase thus allowing AI-2 internalization. Both SirA/BarA and *lsr* regulatory systems are involved in biofilm formation in *S.* Typhimurium.

In agreement with previous reports[152], we determined that deletion of *sirA*, *barA*, *csrA* and *csrBC* resulted in reduced biofilm formation. Additionally, we determined that deletion of *lsrR*, *lsrD* and *barA-ackA-pta* resulted in impaired biofilm formation whereas lack of *ackA-pta* resulted in increased biofilm formation, indicating possible suppressive role of acetate on formation of biofilm. These data suggest common function between SirA/BarA and *lsr* regulatory systems.

A model for SirA/BarA-LsrR interaction which summarizes our results and those from other studies is shown in figure 13. During the early growth phase CsrA suppresses *lsrACDBFGE* translation and thus prevents AI-2 internalization. During the mid-exponential growth phase, the response regulator SirA induces the expression of CsrB and CsrC which sequester repressor protein CsrA which allows the translation of *lsrACDBFGE* operon. At the same time, CsrA prevents LsrR translation and thus de-represses *lsrACDBFGE*.

This study illustrates the complexity of molecular mechanisms involved in the regulation of QS in *S.* Typhimurium. These findings expand our knowledge about regulation of AI-2 QS through discovery of cross-talk between SirA/BarA-CsrA regulatory cascade and *lsr* regulatory system.

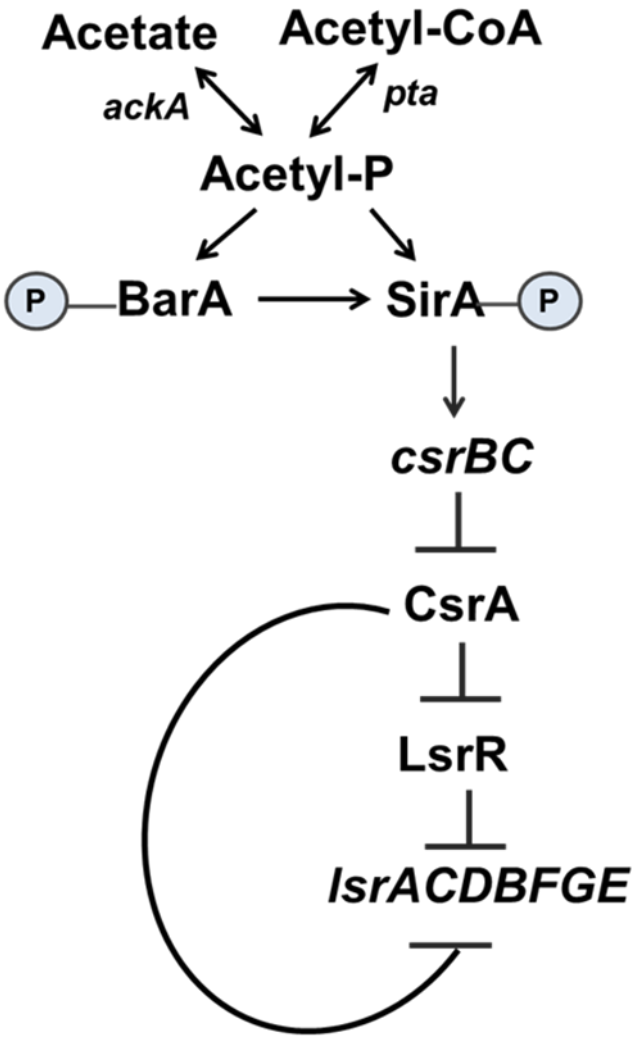

Figure 13: Model of SirA/BarA-CsrA regulation of *lsr*.

2.5. Materials and methods

Primer sequences and bacterial strains used in this study are listed in Tables 1-4.

Table 1: Primers for gene deletion, mutant validation and RNA nucleotides.

Primer	Sequence (5' - 3')	Target gene
Primer for gene deletion		
STM2826 csrA 5'+P1	CAGAGAGACCCGACTCTTTTAATCTTTCAAGGAGCAAAGAGTGTAGGCTGGAGCTGCTTC	*csrA*
STM2826 csrA 3'+P2	GAGAGGCTGCTGCTCACCCGAAAAGTGAGACGCGAAAACCCATATGAATATCCTCCTTAG	
STM2966 csrB 5'+P1	AAGACAAGGTGAAACAGGCGATTCTATCTTCGTCGACAGGGTGTAGGCTGGAGCTGCTTC	*csrB*
STM2966 csrB 3'+P2	GTTAAAAACCTCAATGATGAAAATCTGGCGCGAAGAATAACATATGAATATCCTCCTTAG	
csrC 5' +P1 pcr	GTATCTTGTGAGTTTACCCCAAAAGAGTAAAGTAATGCACTGTAGGCTGGAGCTGCTTC	*csrC*
csrC 3' +P2 pcr	CCGTTTTATTCAGTATAGAATTGAGGCGGAATCTAGCAGACATATGAATATCCTCCTTAG	
STM1947 sirA 5'+P1	TAACTATCAGTAGCGTTATCCCTATTCTGGAGATATTCCTGTGTAGGCTGGAGCTGCTTC	*sirA*
STM1947 sirA 3'+P2	GTAACGGTTTTCAAAAACGCCTTTGCGTCAAATATTTCACCATATGAATATCCTCCTTAG	
barA/STM2958 1016 12 5'+P1	ATTTAACAGTGTGACCCTAATTGTCCCATAACGGAACTCCGTGTAGGCTGGAGCTGCTTC	*barA*
barA/STM2958 1016 12 3'+P4	GTAGGCCGGATAAGGCGGTCACGCCGCCATCCGGCGAATACATATGAATATCCTCCTTAG	
STM2817 luxS 5'+P1	GAGTTCAGTTTATTTTTAAAAAATTATCGGAGGTGACTAAGTGTAGGCTGGAGCTGCTTC	*luxS*
STM2817 luxS 3'pcr+P2	CCGGGGTTAATTTAAATACTGGAACCGCTTACAAATAAGACATATGAATATCCTCCTTAG	
STM4073 LsrR 5'+P1	AATGATTTAGAAATGTTCAAAGTAAAGCCAGGTTATGACAGTGTAGGCTGGAGCTGCTTC	*lsrR*
STM4073 LsrR 3'+P2	TTATTGTTTATTGAATTGAGGTAAGTGTTATTTTATTTTTCATATGAATATCCTCCTTAG	
STM4076 ydeZ 5'+P1	CAAAAACGCACGCACAACAGAATAAAAACAAAGAGGTGGCGTGTAGGCTGGAGCTGCTTC	*lsrD*
STM4076 ydeZ 3'+P2	GTGTCTTGCCCATTTTTTTCTCCATAAATTTCAAAAGGAAACATATGAATATCCTCCTAG	
Primers used to validate deletion and transcription mutants		
STM2826 csrA 5'pcr	TGATGGATAATGCCGGGATA	*csrA*
STM2826 csrA 3'pcr	ACAACGCGGGCGAGCGAGAA	
STM2966 csrB 5'pcr	ATTGTCTGTAAGCGTCTTGT	*csrB*
STM2966 csrB 3'pcr	ACACGGTCATGAGTCGTCAT	
csrC 5'pcr	AAGGCCTCGTCTCCGTCAGTCAAAG	*csrC*
csrC 3'pcr	CCCAAGCTTGAGGCGGAATCTAGCAG	
STM1947 sirA 5'pcr	CCAGCTAATATAAGGACGAT	*sirA*
STM1947 sirA 3'pcr	GATAGACACCGGGTTGGCTG	
barA/STM2958 1016 12 5'pcr	CATCGACCAGCTTTTTCTCA	*barA*
barA/STM2958 1016 12 3'pcr	GGCAACAGTTACGCTGAGTT	
ackA STM2337 5'pcr	GCGCTACGCTCTATGGCTCA	*ackA*
ackA STM2337 3'pcr	TTACCGTCTTTTGGGGACGG	
pta STM2338 5'pcr	TCGCTGGCGGTGCTGTTTTG	*pta*
pta STM2338 3'pcr	GTATCTCACCTGTAGGCCGG	
STM2817 luxS 5'pcr	CAGACTCGCCCCGGAACAAA	*luxS*
STM2817 luxS 3'pcr	TAAAAAACCCCGGCCATAAA	
STM4073 lsrR 5'pcr	GATAACTGAACAATTGTGTT	*lsrR*
STM4073 lsrR 3'pcr	GTTACATAAAATCCTGTCAG	
STM4076 ydeZ 5'pcr	CACTCCACTACAGACGGAAG	*lsrD*
STM4076 ydeZ 3'pcr	AAGGCGATCATTTTAATGCT	
C1	TTATACCAAGGCGACAAGG	
C2	GATCTTCCGTCACAGGTAGG	
k1	CAGTCAATAGCCGAATAGCCT	
k2	CGGTGCCCTGAATGAACTGC	
kt	CGGCCACAGTCGATGAATCC	
Lac	GACCATTTTCAATCCGCA	
Km	TTTCTAGAGCTGTTAAAAGGACA	

Table 2: Primers for T7 promoter.

Primer	Sequence (5' - 3')
T7 promoter primers	
lsrA promoter F	CGAAATAATACGACTCACTATAGGG TGTCATAACCTGGCTTTACT
lsrA promoter R	GGAACCAGGTAAATGCCATA
lsrR promoter F	CGAAATAATACGACTCACTATAGGG GTTGCCTCCGCTCCCTCAATG
lsrR promoter R	GAAGCGGGAGTTGATTTG
hilDFBamHI	TCACAGCCGTTCAGTGAG
hilDRHindIII_T7	CGAAATAATACGACTCACTATAGGGATCTGCGGCAGGACGC

Table 3: Primers used for creating deletion mutants.

Primer	Sequence (5' - 3')	Target gene
Primer for gene deletion		
STM2817 luxS 5'+P1	GAGTTCAGTTTATTTTTAAAAAATTATCGGAGGTGACTAAG TGTAGGCTGGAGCTGCTTC	*luxS*
STM2817 luxS 3'pcr+P2	CCGGGGTTAATTTAAATACTGGAACCGCTTACAAATAAGAC ATATGAATATCCTCCTTAG	
STM4076 ydeZ 5'+P1	CAAAAACGCACGCACAACAGAATAAAAACAAAGAGGTGGC GTGTAGGCTGGAGCTGCTTC	*lsrD*
STM4076 ydeZ 3'+P2	GTGTCTTGCCCATTTTTTTCTCCATAAATTTCAAAAGGAAAC ATATGAATATCCTCCTAG	
rbsB P1	CAACAAAAAGCAGTAACAACGACTACAGGACATCTGAAAT GTGTAGGCTGGAGCTGCTCC	*rbsB*
rbsB P2	TTTCTGTCGCCATCAGGCCATAGTTGACCCGTGTCGTTTAC ATATGAATATCCTCCTTAG	
Primers used to validate deletion and transcription mutants		
STM2817 luxS 5'pcr	CAGACTCGCCCCGGAACAAA	*luxS*
STM2817 luxS 3'pcr	TAAAAAACCCCGGCCATAAA	
STM4076 ydeZ 5'pcr	CACTCCACTACAGACGGAAG	*lsrD*
STM4076 ydeZ 3'pcr	AAGGCGATCATTTTAATGCT	

Table 4: Bacterial strains and plasmids.

Strain	Genotype	Reference/Source
***S.* Typhimurium**		
IR715	*14028s S.*Typhimurium Nal^R	(Stojiljkovic, Baumler et al. 1995)
SDL519	*Δ(sirA)::Cam*	This study
SDL688	*Δ(barA)::Cam*	This study
SDL797	*Δ(csrA)::Cam*	This study
RM STM777	*Δ(ackA-pta):: Kan*	(Lawhon, Maurer et al. 2002)
SDL693	*Δ(barA), Δ(ackA-pta)::Cam*	This study
SDL517	*Δ(csrB)::Cam*	This study
SDL590	*Δ(csrC)::Kan*	This study
CA1005	*Δ(csrBC)::Cam*	(Fortune, Suyemoto et al. 2006)
SDL588	*Δ(lsrR)::Cam*	This study
SDL544	*Δ(lsrD)::Cam*	This study
SDL659	*Δ(luxS)::Cam*	This study
SDL745	*lsrR-lacZ*	This study
SDL774	*Δ(sirA) lsrR-lacZ*	This study
SDL775	*Δ(barA) lsrR-lacZ*	This study
SDL776	*Δ(barA), Δ(ackA-pta) lsrR-lacZ*	This study
SDL803	*Δ(csrA), lsrR-lacZ*	This study
SDL969	*Δ(csrBC) lsrR-lacZ*	This study
SDL778	*lsrD-lacZ*	This study
SDL779	*Δ(sirA) lsrD-lacZ*	This study
SDL780	*Δ(barA) lsrD-lacZ*	This study
SDL781	*Δ(barA), Δ(ackA-pta) lsrD-lacZ*	This study
SDL801	*Δ(csrA), lsrD-lacZ*	This study
SDL970	*Δ(csrBC) lsrD-lacZ*	This study
V. harveyi		
BB170	*V. harveyi* strain for AI-2 detection	ATCC
E. coli		
DH5α	Strain for cloning of recombinant protein, Amp^R	Invitrogen
SG13009	Strain for expression of recombinant protein, Kan^R	ATCC
Plasmids	**Description**	**Reference/Source**
pKD3	pANTγ derivative template plasmid containing the chloramphenicol cassette for lambda Red recombination, Cam^R	Datsenko and Wanner, 2000
pKD4	pANTγ derivative template plasmid containing the kanamycin cassette for lambda Red recombination, Kan^R	Datsenko and Wanner, 2000
pCP20	Plasmid expressing FLP recombinase from a temperature-inducible promoter, Amp^R	Datsenko and Wanner, 2000
pKD46	Plasmid expressing lambda Red recombinase	Datsenko and Wanner, 2000
PCE36	The transcriptional fusion plasmid cointaining LacZY and FRT site in orientation A, Amp^R	Ellermeier et al, 2002
pQE70	Expression vector for constructing C-terminal csrA-His fusion, Amp^R	Qiagen

2.5.1. Bacterial strains, plasmids and growth conditions

Salmonella enterica serovar Typhimurium strains used in this study were derived from spontaneously resistant to nalidixic acid ATCC14028 (IR715)[158]. All *Salmonella* strains were grown aerobically at 37°C with aeration in lysogeny broth (LB) containing 0.5% NaCl, 1% tryptone, 0.5% yeast extract), unless indicated otherwise. When necessary, antibiotics were used in the following concentrations: carbenicillin 100 µg/mL, kanamycin 50 µg/mL, chloramphenicol 30 µg/mL (Sigma).

2.5.2. Construction of deletion mutants and strains with lacZ *fusion*

Chromosomal gene deletion mutant strains were generated by the λRed recombinase system as previously described[159]. Briefly, P1 and P2 primers, homologous to the gene of interest with flanking sequences homologous to the pKD4 (Kan^R) and pKD3 (Cam^R) plasmids, were used to amplify the chloramphenicol and kanamycin resistance genes. These PCR fragments were used to replace the gene of interest with chloramphenicol or kanamycin cassette in strain BL21 using the Red-mediated recombination. Phage P22 transduction was used to replace gene of interest with recombinant allele in WT strain of *S.* Typhimurium. When necessary, the antibiotic resistance cassette was removed using FLP recombinase system located on the pCP20 plasmid generating scarred mutant. The Red and FLP plasmid were cured by growth at 37°C. Double mutants were generated by introducing new deletion into the scarred mutant. The *csrA* strain contained second-site suppression that partially restored growth[157]. The method of Ellermeier[160] was used to construct chromosomal *lacZ* fusions. Briefly, the *lsrR* and *lsrD* deletion strains carrying pCP20 plasmid were

transformed with *lac* transcriptional fusion plasmid pCE36. Phage P22 transduction was used to place *lsrR-lacZ* and *lsrD-lacZ* fusions into appropriate mutants.

2.5.3. Construction of strain expressing His-tagged CsrA

To create the construct expressing C-terminus 6 His-tagged CsrA, open reading frame (ORF) sequence of *csrA* was PCR amplified with the primers csrA-6H5 (acatgcatgctgattctgactcgtc) and csrA-6H3 (cgggatccgtaactggactgctggg). The PCR product was further digested with *Sph*I and *Bam*HI and then cloned into the same restriction enzyme sites of pQE70 plasmid (Qiagen).

2.5.4. Assessment of biofilm formation

Detection of biofilm formation was performed as described[22]. Briefly, ON *S.* Typhimurium cultures were diluted 1:100 in appropriate media and 100 µl of the aliquots were added to round-bottom 96-well plate (Corning) and incubated at 37°C without agitation. Incubation was stopped at 36 h post-inoculation. Plates were washed with ddH_2O and stained for 15 min with 0.1% crystal violet (Azer Scientific). After staining plates were washed with ddH_2O. Ethanol (95%) was added to the wells to dissolve biofilm-associated dye. After thoroughly mixing the content of the well was transferred to optically clear flat-bottom 96-well plate (Corning), and OD_{590} was measured (Synergy2, Biotek).

2.5.5. β-Galactosidase assays

Quadriplicate cultures of each strain were grown with aeration in LB broth at 37°C and β-galactosidase activity was determined as described previously[161]. Briefly, cultures were grown in LB broth to reach OD_{600} of 0.45-0.5, 0.6-0.7 and 0.9-1.0 (early-,

mid- and late-exponential growth phase respectively). Due to naturally low expression levels of *lsrR-lacZ* and *lsrD-lacZ*, cultures were mixed with Z-buffer in ratio 1:1 (500 µl bacterial culture and 500 µl Z-buffer). Z-buffer composition is following: 60 mM $Na_2HPO_4 \bullet 7H_2O$, 40mM $NaH_2PO_4 \bullet H2O$, 10mM KCl, 1mM $MgSO_4 \bullet 7H_2O$ and 0.27% β-Mercaptoethanol. Samples lysed with 100 µl of chloroform and 50 µl of 0.1% SDS. Lysed cultures were incubated with ortho-nitrophenyl-*β*-galactoside (ONPG) at 28°C until sufficient yellow color developed. Reaction was stopped by adding 500 µl of 1M Na_2CO_3 and OD_{420} was measured in spectrophotometer (Genesys 10vis, Thermo scientific). Beta-galactosidase units were calculated by formula: β-gal= $\frac{1000*OD420}{T*V*OD600}$, where T = time in minutes, V= volume of bacterial culture in milliliters.

2.5.6. Autoinducer-2 assay

AI-2 assay was performed as previously described[162]. Briefly, overnight *Salmonella* cultures were diluted 1:100 and grown with aeration in LB broth. At determined time points 500 µl of culture was taken, centrifuged for 3 min at 7000 g and filtered through 0.2 µm filter to generate cell-free supernatants. Frozen stock of *V. harveyi* strain BB170 was inoculated into 5 mL of AB medium (17.5 g NaCl, 12.3 g MgSO4, 2.0 g Casamino acids (Difco, MI) per 1 L of water, pH 7.5. Sterilize, cooled down, and add 10 mL of 1 M KPi (pH 7.0), 10 mL of 0.1 M L-arginine and 10 mL of Glycerol[163] and grown for 16 h to reach OD_{600} =~0.7 to 1.2). *V. harveyi* culture was diluted 1: 5000 in AB media and 10 µl of cell-free *Salmonella* supernatants was added to 90 µl of *V. harveyi* culture. Samples were incubated with aeration at 30°C on the black well 96-well plate (Corning). Luminescence was measured every hour (at 490 nm) until

negative control (*V. harveyi* cultures incubated without *Salmonella* supernatants) has produced the lowest amount of light.

2.5.7. Protein extraction

CsrA extraction followed published procedure[150b]. Briefly, 50 mL of *E. coli* strain SG13009 containing pQE70 plasmid carrying csrA-His on the C-terminus was grown in LB to reach OD_{600} of 0.6. Then 1 mM of IPTG was added to the culture and incubated for additional 6h. Bacterial pellets were collected, and re-suspended in 8 mL of lysis buffer (50 mM NaH_2PO4, 500 mM NaCl pH = 8.0, 10% glycerol, 0.1% Tween 80 and 1 mg/mL lysozyme) for 30 min on ice. Then cells were aspirated 6-10 times through 18 g needle, centrifuged at 3000 × g for 15 min and supernatant was filtered through gauze and applied on 2 mL nickel column (Nickel-Chelating Resin, Life Technologies) for 1 h at 4°C. Column was washed 2 times with washing buffer (50 mM NaH_2PO_4, 50 mM NaCl pH = 8.0, 10% glycerol) and eluted with washing buffer containing 20 mM Imidazole. Fractions containing pure protein were pulled together and dialyzed against 10 mM Tris-HCl, pH 7.0, 100 mM KCl, 10 mM $MgCl_2$ and 25% glycerol. Lack of protein aggregation was confirmed by dynamic light scattering (Malvern Instruments Ltd, UK).

2.5.8. RNA synthesis and labelling

Antisense HilD promoter RNA was synthesized using MEGAscript T7 Transcription Kit (ThermoFisher scientific). Briefly, DNA template was amplified via PCR and incubated for 16 h at 37°C with ATP, GTP, CTP, GTP, UTP, buffer and enzyme mix. Then mixture was treated with TURBO DNase, RNA was precipitated with

Lithium Chloride, supernatant was removed and pellet was washed with 70% ethanol. Then pellet was resuspended in water. RNA was labelled with Pierce RNA 3' End Biotinylation Kit. Briefly, 50pmol of RNA was incubated in ligation reaction containing 10 µL, 0.05M Tris-HCl, 0.01 M $MgCl_2$, 0.01 M DTT, 1 mM ATP; pH 7.8, 1.33 U RNase inhibitor, 33.3 µM biotinylated cytidine (Bis) phosphate, 1.33 U T4 RNA Ligase and 15% PEG for 2 h at 16°C. Then reaction was stopped by adding water; RNA ligase was extracted with chloroform : isoamyl alcohol, RNA was precipitated with 10 µL of 5 M NaCl, 1 µL of glycogen, and 300 µL of ice-cold 100% ethanol for 2 hours at -20°C, centrifuged for 15 minutes at 13,000 × g at 4°C. Pellet was washed with ice cold 70% ethanol, air-dried and resuspended in nuclease-free water. LsrR, LsrA and HilD promoter biotinylated oligonucleotides were commercially synthesized (Sigma-Aldrich).

2.5.9. DRaCALA

Differential radial capillary action of ligand assay (DRaCALA) was employed to determine the interaction between CsrA and *lsr* promoter[164]. Various concentrations of CsrA were incubated with 500 pM of *lsrR* and *lsrA* promoter RNA. HilD sense and antisense RNA were served as positive and negative control respectively. Prior to reaction RNA was heated for 5 min at 80°C and then cooled down on ice. Binding buffer contained 10 mM Tris pH = 7.5, 10mM $MgCl_2$, 100 mM KCl, 7.5% glycerol and 20 mM DTT and binding reaction volume was 10 µL, reactions were set in triplicates. Binding reactions were incubated for 30 min at 37°C. Then 2 µL of reaction was spotted on dry nitrocellulose membrane and cross-linked with FB-UVX-100 UV-crosslinker (Fisher). Membranes were developed with Pierce kit (Life Technologies) according to

manufacturer's instruction and exposed for 2 min to X-ray film. ImageJ[165] was used for intensity quantifications. The relative affinity of protein-ligand binding was calculated as fraction bound (F_B) according to published procedure[164].

2.5.10. Statistical analysis

ANOVA followed by Tukey's T-test was performed (JMP, Version 11. SAS Institute Inc., Cary, NC, 1989-2007) to determine the difference in extracellular AI-2 concentrations and gene expression.

3. INTERACTION BETWEEN AI-3/EPI/NE AND AI-2 QUORUM SENSING SYSTEMS IN *SALMONELLA* TYPHIMURIUM

3.1. Summary

In *Salmonella* Typhimurium the BarA/SirA-*csrBC*-CsrA-LsrR regulatory cascade regulates autoinducer 2 (AI-2) quorum sensing (QS) while QseBC and QseEF systems regulate AI-3 signaling. Both AI-2 and AI-3 QS systems regulate virulence (SPI-1) and motility in *S.* Typhimurium. It has been determined via microarray that the expression of *lsrADR* and *csrB* was decreased when *E. coli* was grown with epinephrine (Epi) and norepinephrine (NE). The QseBC/EF system is involved in AI-3/NE/Epi signaling in *E. coli* and *S*. Typhimurium. However, the interaction between QseBC/EF and *lsr* systems is not known. The goal of this study is to determine whether there is a cross-talk between the *lsr* and AI-3/Epi/NE QS systems. Here we present an evidence of an interaction between AI-2 and AI-3/NE/Epi signaling. We determined that QseF regulates *lsrR* and *lsrD* expression, as well as extracellular AI-2 levels during the mid-exponential growth phase. Additionally, catecholamine NE decreased *lsrR* expression and extracellular AI-2 levels. Therefore we propose that there is an interaction between AI-2 and AI-3/Epi/NE signaling systems where QseF- and NE-dependent signaling regulates the activity of *lsr* operon in *S.* Typhimurium.

3.2. Introduction

Bacteria need to continuously adapt to the environment to colonize the gut. Quorum sensing is a method of bacterial communication used to regulate gene

expression in bacterial population. The enteric pathogen *Salmonella* Typhimurium employs quorum sensing order to regulate virulence and outcompete the resident microbiota[1]. Quorum sensing molecule autoinducer 2 (AI-2) production is a produced due to series of enzymatic reactions, ending in methylthioadenosine/S-adenosylhomocysteine nucleosidase and S-ribosylhomocysteinase (LuxS) converting SAH to 4,5 dihydroxy-2,3-pentanedione (DPD), which is cyclized into (2R, 4S)-2-methyl-2,3,3,4-tetrahydroxytetra-hydrofuran (AI-2) intracellularly[18]. The majority of AI-2 is produced and released during exponential growth[18a] and is transported into the cell via the ATP transporter encoded on *lsr* operon by *lsrACDB*[20]. Inside the bacterial cell AI-2 is phosphorylated[18a] and inactivates the transcriptional repressor protein LsrR[20] and induces *lsr*ACDBFGE operon transcription[18a] thus further increasing AI-2 internalization. In *S.* Typhimurium, LsrR negatively regulates expression of genes of *lsr*ACDBFGE operon and itself. LsrR also suppresses expression of flagella (*fliC, fliD*) and genes encoded on *Salmonella* pathogenicity island 1 (SPI-1) which regulates invasion (*sipC*) and oxidative stress response (*sodA, sodCI, sodCII*)[23-24]. SipC promotes actin nucleation and intestinal inflammation and is essential for pathogen internalization [166].

Salmonella Typhimurium also encodes the two-component regulatory systems QseC/B and QseF/E[33 34]. The histidine sensor kinase QseC can recognize autoinducer 3 (AI-3) and the catecholamines epinephrine (Epi) and norepinephrine (NE), autophosphorylates, and subsequently dephosphorylates the response regulator QseB thus inducing SPI-2 gene expression[36], motility and invasion and survival in

macrophages[36b]. The sensor kinase QseE senses NE, Epi, phosphate and sulfate and activates the response regulator QseF, thereby inducing the expression of SPI-1 encoded genes. QseC-dependent signaling can be blocked by the α-adrenergic antagonist phentolamine[37]; however, the effect of adrenergic antagonists on QseE is unclear. Host stress hormones NE and Epi have been shown to induce growth of *S.* Typhimurium in iron-restricted media[34, 38] and increase the expression of genes encoded on SPI-1 (*sipA, sipB*) and SPI-3 (*mgt*)[36b] as well as increase plasmid transfer[41]. The debate is whether the NE-dependent enhancement of bacterial growth, motility and virulence depends on QseC/E signaling[34, 36b]. In *V. cholera*e, motility and expression of *qseC* and *pomB* (structural component of the flagellar motor complex) was enhanced in the presence of Epi and NE[167]. It has also been shown that the alpha-adrenergic antagonist, phentolamine, and the beta-adrenergic antagonist, propranolol, can neutralize norepinephrine-induced enhancement of motility and plasmid transfer[36a, 39, 41], although this has not been universally reported[34].Catecholamine-dependent signaling may be involved in AI-2-dependent virulence regulation in *Enterobacteriaceae* as it has been shown that NE and Epi decrease the expression of *lsrR* and *lsrACDB* in *E. coli* O157:H7[42] suggesting an interaction between AI-3/Epi/NE and *lsr* regulation systems. However, it is unknown whether the AI-3/Epi/NE signaling system is involved in the regulation of AI-2 QS in *Enterobacteriaceae.* The primary objective of this study was to evaluate a role of the AI-3/Epi/NE signaling system and NE in regulation of *lsr*-dependent AI-2 QS in *S.* Typhimurium.

3.3. Results

3.3.1. QseB/C and QseE/F do not regulate luxS *expression in* S. *Typhimurium*

It has been shown that deletion of *luxS* affects expression of multiple genes in *Salmonella*[156], however, AI-3 synthesis does not depend on *luxS*[32b]. It is unclear whether AI-3-dependent QS mechanisms through qseB/C and qseE/F affect *luxS* expression in *Salmonella*. Thus we assessed *luxS-lacZ* expression in strains deficient in AI-3 quorum sensing. Mutants were lacking a sensor kinase (*qseC, qseE*), or response regulator (*qseB, qseF*) or both (*qseBC, qseEF*). The deletion of the AI-3/Epi/NE signaling system did not alter *luxS* expression (P= 0.291) as determined by beta-galactosidase *luxS-lacZ* assay (Fig. 14).

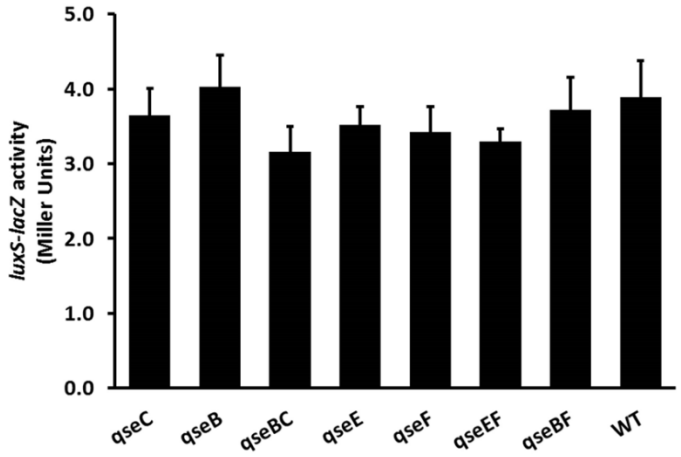

Figure 14: *LuxS-lacZ* expression is not regulated by the AI-3 quorum sensing system. *β*-galactosidase activity of the *luxS-lacZ* was determined in WT, *qseB, qseC, qseBC, qseE, qseF* and *qseEF* strains at mid-exponential growth phase. Bars represent standard deviation. *$P < 0.05$ when compared to WT.

3.3.2. QseF regulates the expression of lsr *operon in* S. *Typhimurium*

The expression of the *lsr* operon is known to be regulated by repressor protein LsrR, which binds to promoters of *lsrKR* and *lsrACDBEFG* and repressed the transcription of AI-2 transporter and itself. To determine if QseBC and QseEF are involved in the regulation of *lsr* operon we assayed the expression of *lsrR-lacZ* and *lsrD-lacZ* in *qseB, qseC, qseBC, qseE, qseF* and *qseEF* deletion mutants in comparison to WT. Deletion of response regulator *qseF* and both response regulators *qseB* and *qseBF* resulted in increased *lsrR-lacZ* (Fig. 15A) and *lsrD lacZ* (Fig. 15B) expression at mid-exponential growth phase. This indicates that the AI-2 transporter as well as the *lsr* repressor is regulated by AI-3/Epi/NE signaling system.

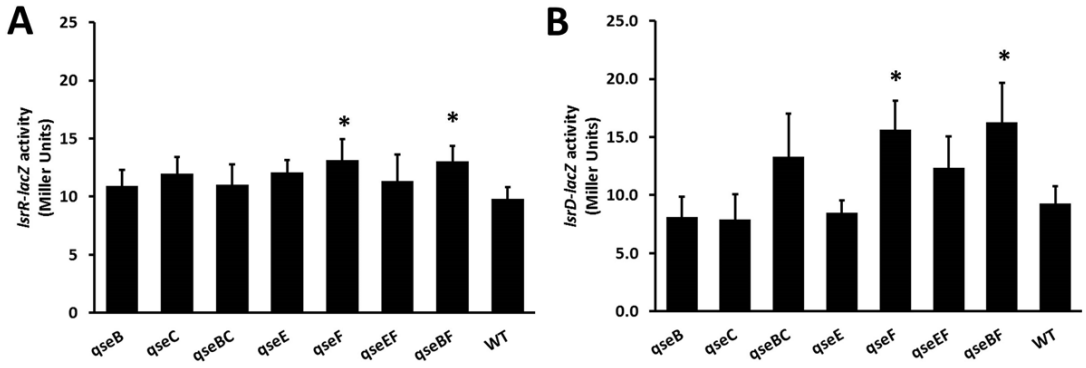

Figure 15: QseF regulates *lsrR-lacZ* and *lsrD-lacZ* expression. Bars represent standard deviation. *$P < 0.05$ when compared to WT. β-galactosidase activity of the *lsrR-lacZ* (A) and *lsrD-lacZ* (B) was measured in WT, *qseB, qseC, qseBC, qseE, qseF* and *qseEF* strains at mid-exponential growth phase.

3.3.3. AI-3/Epi/NE signaling system regulates extracellular AI-2 levels

The *lsr* operon of *S.* Typhimurium regulates the internalization (*lsrACDB*) of extracellular AI-2 as well as its further phosphorylation (*lsrK*) and degradation

(*lsrFG*)[141b, 142, 168]. The efficiency of AI-2 internalization can be assessed indirectly by measuring levels of extracellular AI-2. Increased extracellular levels imply decreased AI-2 transporter activity whereas decreased extracellular AI-2 levels suggest increased internalization. The extracellular AI-2 levels were decreased in *qseB* and *qseBF* mutant (Fig. 16), suggesting that AI-3/Epi/NE signaling system decreases activity of the AI-2 transporter. We have determined that QseB/C and QseE/F do not affect *luxS* expression, suggesting no effect on AI-2 production. Therefore, altered AI-2 levels in *qseF* and *qseBF* mutants are likely due to changes in AI-2 internalization.

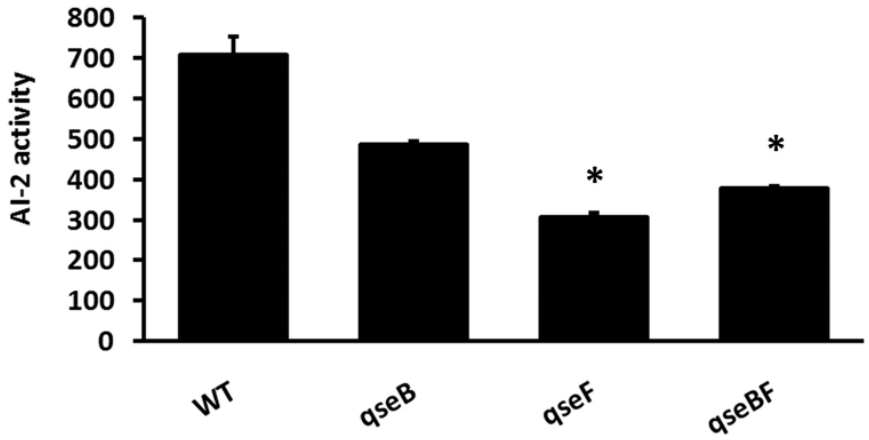

Figure 16: QseF regulates extracellular AI-2 levels. Bars represent standard deviation. *$P < 0.05$ when compared to WT. Cell-free supernatants were collected from WT, *qseB, qseC, qseBC, qseE, qseF* and *qseEF* strains at mid-exponential growth phase.

3.3.4. Norepinephrine regulates lsrR *expression*

Bansal *et. al.*[42] determined that 50 µM NE decreased *lsrR* expression in *E. coli.* It is known that QseC and QseE recognize NE[37, 169], and contribute to NE-induced virulence[36b, 170]. Since NE regulates gene expression through QseBC and QseEF, we

inquired whether NE regulates *lsr* expression in *S.* Typhimurium. The addition of 50 µM of NE, which exceeds physiological gut NE concentration [13b], decreased expression of *lsrR-lacZ* at mid-log growth phase (Fig. 17A). The expression of invasion, SPI-1-encoded gene *sipC* is negatively regulated by LsrR[24]. We determined that incubation of *S.* Typhimurium with 50 µM NE increased *sipC-lacZ* expression (Fig. 17B), suggesting that NE negatively regulates *lsrR* expression at mid-log growth phase. Additionally, we determined that NE did not affect *sipC-lacZ* expression in a *lsrR* deletion mutant (Fig. 16B). Together, this data suggests that NE regulates *lsrR* expression in *S.* Typhimurium.

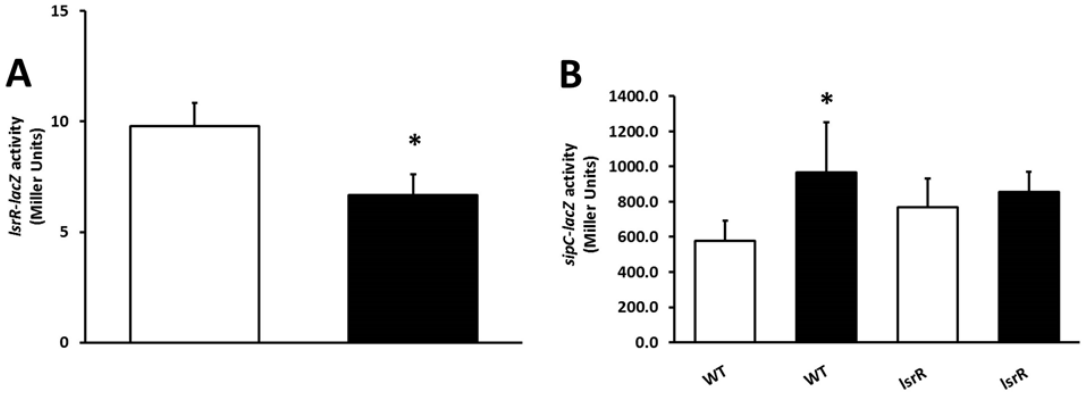

Figure 17: NE regulates *lrsR* and *sipC* expression. Bars represent standard deviation. *$P < 0.05$ when compared to (-)NE. *β*-galactosidase activity of the *lsrR-lacZ* (A) was determined in WT; *β*-galactosidase activity of the *sipC-lacZ* (B) was determined in WT and *lsrR* deletion mutant. *lsrR-lacZ* and *sipC-lacZ* activity was measured at mid-exponential growth phase.

3.3.5. Norepinephrine decreases extracellular AI-2 concentrations in S. *Typhimurium*

By the mid-log growth phase extracellular AI-2 reaches high concentration and is actively transported into the cell via *lsrACDB*. To further elucidate the effect of AI-3/Epi/NE regulatory system on the *lsr* operon, we measured extracellular AI-2 levels in

NE-treated cultures. First, we determined that 50 µM of NE decreased extracellular AI-2 concentration in WT *Salmonella* (Fig. 18A), suggesting increased AI-2 internalization in the presence of NE. To determine if the effect of NE is through AI-3/Epi/NE signaling system, we incubated *Salmonella* with the adrenergic antagonist phentolamine. Phentolamine has been shown to reverse NE-induced motility[171] and plasmid transfer[41] as it signals through QseBC. Surprisingly, incubation with 200 µM phentolamine decreased extracellular AI-2 compared to 50 µM NE (Fig. 18A). Co-incubation with NE and phentolamine restored extracellular AI-2 levels to those when incubated with NE only (Fig. 17A). In a *lsrD* mutant, incubation with NE or co-incubation with NE and phentolamine (Fig. 18B) did not affect extracellular AI-2 activity, however, 200 µM phentolamine decreased extracellular AI-2 (Fig. 18B). This data suggests that NE regulates extracellular AI-2 levels through AI-3/Epi/NE signaling.

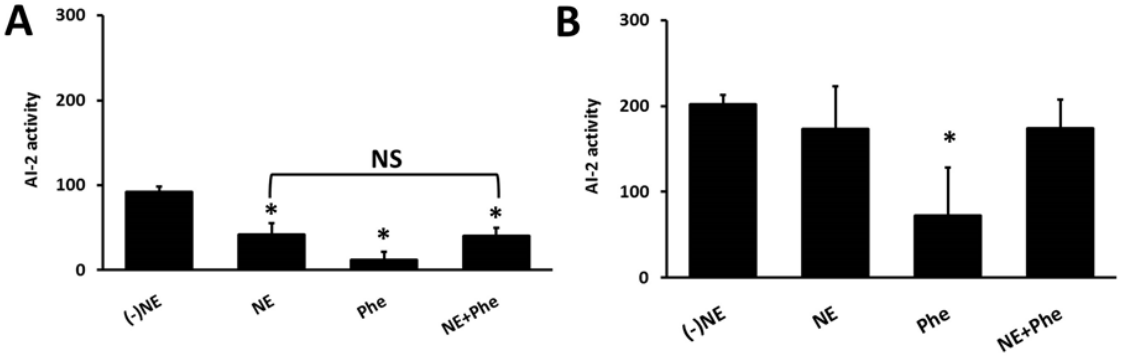

Figure 18: Effect of norepinephrine and phentolamine on extracellular AI-2 in WT (A) and *lsrD* (B) strains. Extracellular AI-2 was measured at mid-exponential growth phase. *$P < 0.05$ when compared to (-) NE.

3.4. Discussion

Bacterial quorum sensing aids pathogenic bacteria in virulence and colonization efficiency[125, 171]. Stress in the host increases the risk of infection by pathogenic microbes[128, 131]. In *S.* Typhimurium, adrenergic QseC and QseE receptors can recognize host-derived catecholamines Epi and NE in addition to self-produced AI-3[35]. It has been shown that AI-3, NE and Epi increased virulence by regulating SPI-1 and SPI-2 gene expression[35]. The AI-2 QS system also regulates virulence in *S.* typhimurium[24], while NE and Epi decrease expression of the *lsr* operon, which encodes for AI-2 transporter (*lsrACDB*) and SPI-1 gene repressor (*lsrR*)[42]. Previous studies suggested that *luxS* regulates the AI-3/Epi/NE signaling system[32a, 172]; however, observed changes in QseBC expression were due to decreased AI-3 synthesis in *luxS* mutant, but not due to AI-2 loss[32b]. It was shown that QseEFG transcriptionally regulate the PhoPQ and RcsBC two-component systems in *E. coli* O157:H7[173] indicating the presence of a cross-talk between catecholamine (QseEFG)and antimicrobial peptide (PhoPQ) sensing as well as expression of the LEE region of *E. coli* (RcsBC).

The question still remains whether there is a cross-talk between AI-2 and AI-3 QS systems in *S.* Typhimurium. In this study we observed that AI-3/Epi/NE signaling system regulates *lsr* –dependent QS in *S.* Typhimurium. In particular, the QseF response regulator suppressed *lsrR* and *lsrD* expression. Extracellular AI-2 concentrations decreased in *qseF* mutant compared to WT, suggesting that QseF-regulated changes in the expression of *lsr* operon alter extracellular AI-2 concentrations. QseF is a response regulator which is activated when its sensor kinase QseE encounters extracellular stimuli

such Epi, NE, sulphate and phosphate but also can be phosphorylated by QseC directly[35].

We observed QseF-dependent changes in extracellular AI-2 levels, where AI-2 was decreased in *qseF* and *qseBF* mutant. One can speculate that observed changes in *lsr* expression and AI-2 levels were due to changes in AI-2 production, however we demonstrated that *luxS* expression was not dependent on *qseBC* or *qseEF* expression. Multiple reports addressed an importance of interkingdom signaling for bacterial virulence. Host stress hormones Epi and NE have been shown to regulate virulence of *S.* Typhimurium[131], *E. coli* O157:H7[42], *Citrobacter rodentium*[170] and *Vibrio cholerae*[167]. A recent study has shown that colonization levels of *C. rodentium* were higher in Dbh +/- mice compared to catecholamine deficient Dbh-/- mice, suggesting that epinephrine and norepinephrine profoundly alter infection *in vivo*[170].

In this study we have presented evidence that the physiological concentration of NE (50 μM) regulates *lsr*-dependent QS by decreasing *lsrR* expression. To support this claim we demonstrated that NE increases expression of the *sipC* invasion gene, which is normally suppressed by LsrR. Thus NE relieves *sipC* expression by inhibiting *lsrR*. Additionally, NE decreased extracellular AI-2 levels in WT *Salmonella*, suggesting an NE-dependent increases in AI-2 internalization.

There is a controversy regarding α and β adrenergic receptors to catecholamines in Enterobacteriaceae. Previous studies have demonstrated that the α and β adrenergic antagonists phentolamine and propranolol, respectively, decreased expression of virulence genes[32a, 41], while other groups only demonstrated the inhibitory effect through

α receptors only[38]. However, since QseC activates expression of the *qseEF* genes, being at the top of this signaling cascade[174], the inactivation of QseC by phentolamine should lead to the alteration of QseB and QseF signaling. In present study we expected that addition of α adrenergic antagonist phentolamine would reverse the effect of NE on AI-2 levels. Surprisingly, treatment with phentolamine only decreased extracellular AI-2 compared to NE treatment. Simultaneous incubation with NE and phentolamine restored AI-2 levels to those seen in bacteria treated with NE only.

This study demonstrates the evidence of cross-regulation of two quorum sensing systems in the enteric pathogen *S*. Typhimurium, and it also demonstrates the effect of interkingdom communication on the expression of the *lsr* system, virulence genes (*sipC*) and extracellular AI-2 concentrations. This shows that not only QS systems regulate same processes in the cell, but that QS systems regulate each other, and that mammalian stress hormones regulate bacterial QS (AI-2). This study accentuates the importance of bacterial communication in the gut, and the importance of interkingdom cross-talk between the pathogen and the host.

3.5. Materials and methods

3.5.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used are listed in Table 5. *Salmonella enterica* serovar Typhimurium strains used in this study were derived from spontaneously resistant to nalidixic acid ATCC14028 (IR715)[158]. All *Salmonella* strains were grown aerobically at 37°C with aeration in lysogeny broth (LB) containing 0.5% NaCl, 1% tryptone, 0.5% yeast extract (BD, Franklin, NJ), unless indicated otherwise. When

necessary, antibiotics were used in the following concentrations: carbenicillin 100 µg/mL, kanamycin 50 µg/mL, chloramphenicol 30 µg/mL (Sigma-Aldrich, Inc., St. Louis, MO). Additionally, norepinephrine (50 µM) (Sigma-Aldrich, Inc.) and phentolamine (200 µM) (Sigma-Aldrich, Inc.) were added to the cultures when necessary.

3.5.2. Construction of deletion mutants and strains with lacZ *fusion*

Chromosomal gene deletion mutant strains were generated by the λRed recombinase system as previously described[159]. Primers are listed in table 6. Briefly, P1 and P2 primers, homologous to the gene of interest with flanking sequences homologous to the pKD4 (KanR) and pKD3 (CamR) plasmids, were used to amplify the chloramphenicol and kanamycin resistance genes. These PCR fragments were used to replace the gene of interest with chloramphenicol or kanamycin cassette in strain BL21 using the λRed-mediated recombination. Phage P22 transduction was used to replace gene of interest with recombinant allele in the wild type (WT) strain of *S*. Typhimurium. When necessary, the antibiotic resistance cassette was removed using FLP recombinase system located on the pCP20 plasmid generating a scarred mutant. The λRed and FLP plasmid were cured by growth at 37°C. Double mutants were generated by introducing new deletion into the scarred mutant. The *csrA* strain contained second-site suppression that partially restored growth (Altier et al., 2000a). LacZ fusions were constructed using previously published method (Ellermeier et al., 2002). Briefly, the *lsrR*, *lsrD* and *sipC* deletion strains carrying pCP20 plasmid were transformed with *lac* transcriptional fusion

plasmid pCE36. Phage P22 transduction was used to transfer *lacZ* fusions into appropriate mutants.

3.5.3. β-Galactosidase assays

Salmonella cultures were grown with aeration in LB broth at 37°C and β-galactosidase activity was determined as described previously[161]. Due to naturally low expression levels of *lsrR-lacZ* and *lsrD-lacZ*, cultures were mixed with Z-buffer in ratio 1:1 (500 µl bacterial culture and 500 µl Z-buffer). For *sipC-lacZ* expression 900 µl of Z-buffer was mixed with 100 µl of the culture. Z-buffer composition is following: 60 mM $Na_2HPO_4 \bullet 7H_2O$, 40mM $NaH_2PO_4 \bullet H_2O$, 10mM KCl, 1mM $MgSO_4 \bullet 7H_2O$ and 0.27% β-Mercaptoethanol. Samples were lysed with 100 µl of chloroform and 50 µl of 0.1% sodium dodecyl sulfate (SDS). Lysed cultures were incubated with ortho-nitrophenyl-β-galactoside (ONPG) at 28°C until sufficient yellow color developed. Reaction was stopped by adding 500 µl of 1M Na_2CO_3 and OD_{420} was measured in spectrophotometer (Genesys 10vis, Thermo Scientific). β -galactosidase units were calculated by formula: $\beta\text{-gal} = (1000*OD_{420})/(T*V*OD_{600})$, where T = time in minutes, V= volume of bacterial culture in milliliters.

Table 5: Bacterial strains and plasmids used to make deletion mutants and *lacZ* fusions.

Strain	Genotype	Reference/Source
***S.* Typhimurium**		
WT	14028s *Salmonella typhimurium*	ATCC
STM588	Δ(*lsrR*)::Cam	This study
STM544	Δ(*lsrD*)::Cam	This study
STM659	Δ (*luxS*)::Cam	This study
STM745	*lsrR-lacZ*	This study
STM778	*lsrD-lacZ*	This study
STM275	*sipC-lacZ*	This study
STM275	Δ*(lsrR) sipC-acZ*	This study
STM275	*(lsrD) sipC-lacZ*	This study
STM1134	Δ(*qseB*)::Kan	This study
STM1135	Δ(*qseC*)::Kan	This study
STM1159	Δ (*qseBC*)::Cam	This study
STM1142	Δ(*qseE:*Cam	This study
STM1141	Δ(*qseF*)::Kan	This study
STM1160	Δ (*qseEF*)::Cam	This study
STM1158	Δ (*qseBF*)::Cam	This study
V. harveyi		
BB170	*V. harveyi*	ATCC
Plasmids	**Description**	**Reference/Source**
pKD3	pANTγ derivative template plasmid containing the chloramphenicol cassette for lambda Red recombination, Cam^R	Datsenko and Wanner, 2000
pKD4	pANTγ derivative template plasmid containing the kanamycin cassette for lambda Red recombination, Kan^R	Datsenko and Wanner, 2000
pCP20	Plasmid expressing FLP recombinase from a temperature-inducible promoter, Amp^R	Datsenko and Wanner, 2000

Table 6: Primers used to make deletion mutants and *lacZ* fusions.

Primer	Sequence (5' - 3')	Target gene
qseC 5'pcr	CTCGGCAGCGAATTTATTCG	*qseC*
qseC 3'pcr	GGTGAAATTAGCAAAATGTG	
qseC 5'+P1	CACCGTGCACGGCATCGGCTACACCCTGGGTGACGCATGAGTGTAGGCTGGAGCTGCTTC	
qseC 3'+P2	CAAAGTCTTTTGCGTTTTTGGCAAAAGTCTCTGTTACCAA CATATGAATATCCTCCTTAG	
qseB 5'pcr	GTACATCGCCTGCGGCGACAAG	*qseB*
qseB 3'pcr	CCAGAATCAGGAAAATAAGC	
qseB 5'+P1	GTTAACTGACGGCAACGCGAGTTACCGCAAGGAAGAACAG GTGTAGGCTGGAGCTGCTTC	
qseB 3'+P2	GTCAGCCTGACGCGCAGGCTGAGACGTTGCGTCAATTTCAT CATATGAATATCCTCCTTAG	
qseE 5'pcr	GCCCGGCTTTGTTGTATCTG	*qseE*
qseE 3'pcr	GCATCGTTTAATAGTTTGGA	
qseE 5'+P1	GCGCTTCCCTCGGTTAGCATCTTTTTTATTCTTCTTTTATGTGTAGGCTGGAGCTGCTTC	
qseE 3'+P2	CGAAAACGTGTGACATACGCACCAGGCTTAAATTCATA CATATGAATATCCTCCTTAG	
qseF 5'pcr	CCCGCGACGTCTGAAGATGG	*qseF*
qseF 3'pcr	GTGTTTTGATCGGTTAAAC	
qseF 5'+P1	CGCGGCGCCGTCGCCGTCACAAGATGAGGTAACGCCATGA GTGTAGGCTGGAGCTGCTTC	
qseF 3'+P2	GTAACATATTTCGCGCTACTTTACGGCATGAAAA CATATGAATATCCTCCTTAG	
STM2817 luxS 5'pcr	CAGACTCGCCCCGGAACAAA	*luxS*
STM2817 luxS 3'pcr	TAAAAAACCCCGGCCATAAA	
STM2817 luxS 5'+P1	GAGTTCAGTTTATTTTTAAAAAATTATCGGAGGTGACTAAGTGTAGGCTGGAGCTGCTTC	
STM2817 luxS 3'pcr+P2	CCGGGGTTAATTTAAATACTGGAACCGCTTACAAATAAGACATATGAATATCCTCCTTAG	
STM4073 LsrR 5'pcr	GATAACTGAACAATTGTGTT	*lsrR*
STM4073 LsrR 3'pcr	GTTACATAAAATCCTGTCAG	
STM4073 LsrR 5'+P1	AATGATTTAGAAATGTTCAAAGTAAAGCCAGGTTATGACAGTGTAGGCTGGAGCTGCTTC	
STM4073 LsrR 3'+P2	TTATTGTTTATTGAATTGAGGTAAGTGTTATTTTATTTTTCATATGAATATCCTCCTTAG	
STM4076 ydeZ 5'pcr	CACTCCACTACAGACGGAAG	*lsrD*
STM4076 ydeZ 3'pcr	AAGGCGATCATTTTAATGCT	
STM4076 ydeZ 5'+P1	CAAAAACGCACGCACAACAGAATAAAAACAAAGAGGTGGCGTGTAGGCTGGAGCTGCTTC	
STM4076 ydeZ 3'+P2	GTGTCTTGCCCATTTTTTTCTCCATAAATTTCAAAAGGAAACATATGAATATCCTCCTAG	

3.5.4. Autoinducer-2 assay

AI-2 assay was performed as previously described[162]. Briefly, overnight *Salmonella* cultures were diluted 1:100 and grown with aeration in LB broth. At determined time points 500 µl of culture was taken, centrifuged for 3 min at 7000 g and filtered through 0.2 µm filter to generate cell-free supernatants. Frozen stock of *V. harveyi* strain BB170 was inoculated into 5 mL of AB medium[163] and grown for 16 h to reach OD_{600} =~0.7 to 1.2). *V. harveyi* culture was diluted 1: 5000 in AB media and 10 µl of cell-free *Salmonella* supernatants was added to 90 µl of *V. harveyi* culture. Samples were incubated at with aeration at 30°C in a black well 96-well plate (Corning).

Luminescence was measured every hour (at 490 nm) until negative control (*V. harveyi* cultures incubated with 0.5% NaCl LB) has produced the lowest amount of light.

3.5.5. Statistical analysis

ANOVA followed by Tukey's T-test was performed (JMP, Version 11. SAS Institute Inc., Cary, NC, 1989-2007) on log-transformed values to determine the difference in extracellular AI-2 concentrations and gene expression.

4. THE EFFECT OF WEANING STRESS, SEX AND TEMPERAMENT ON FECAL MICROBIOTA AND METABOLITES IN BRAHMAN CALVES

4.1. Summary

A diverse microbial community, also known as microbiota, inhabits the mammalian digestive tract. Composition of the gut microbiota depends on the age, diet and hormonal status of the animal. The gut-brain axis connects the central and peripheral nervous systems, thus regulating immunity, digestion and neuroendocrine signaling. Receptors for cortisol and catecholamines (β adrenergic) are present in various tissues and organs including the brain, gut and microbiota, enabling cross-talk between these organs. In particular, the microbiota is involved in the regulation of the hypothalamic pituitary axis (HPA), thus modulating stress responses of the host. However, the effect of weaning stress, sex classification and temperament on the bovine fecal microbiota and metabolites is not fully understood. The goal of this study was to 1) investigate the effect of weaning stress on the fecal microbiota as well as serum and fecal metabolites in Brahman calves, and 2) compare the fecal microbiota as well as serum and fecal metabolites between males (bulls) and females (heifers) as well as between calm and temperamental animals at weaning (d0) and 4 days post weaning (d4). The PCoA plot showed significant separation between the fecal samples from d0 and d4 after weaning as well as between males and females at d4 after weaning. A total of 50 bacterial taxa were differentially abundant between d0 and d4 after weaning. Additionally, 31 taxa were differentially abundant between males and females at d4 after weaning, and seven

taxa were differentially abundant between temperamental and calm animals at d4 after weaning. Seventy-one and forty-nine metabolites were differentially enriched between d0 and d4 after weaning in serum and feces respectively. Thirty-one taxa and 9 fecal metabolites were differentially abundant between males and females at d4 after weaning. Seven taxa and 5 serum metabolites were differentially enriched between calm and temperamental animals at d4, while 10 serum metabolites were differentially enriched between temperaments at d0 (prior to weaning). Additionally, three serum metabolites were differentially enriched between calm and temperamental males at d0.

These data suggest that weaning has a major impact on microbiota and metabolite composition in Brahman calves. Additionally, sex of the calf and temperament are associated with differentially abundant bacterial taxa and metabolites within a sampling day relative to weaning.

4.2. Introduction

The gut-brain cross-talk, which plays an important role in connecting the central and peripheral nervous systems in order to regulate immunity, digestion and neuroendocrine signaling, involves the central, neuroendocrine, neuroimmune, autonomic and enteric nervous systems. It has been recently recognized that the gut microbiota plays an important role in regulation of the HPA axis, particularly, in modulating the host's response to stress. For example, stress in early life, such as maternal separation, alters behavior, immunity and microbiota in rats[77]. In turn, it is known that the microbiota regulates brain development and metabolism[87]. It has been shown that gut microbiota may regulate the HPA axis and stress responsiveness by

directly regulating neuronal expansion and morphology via expansion of the amygdala and hippocampus[101]. Excitable temperament in animals is associated with increased resting concentrations of cortisol[99] and differential immune response to bacterial infection[100]. Interestingly, in children, temperament (increased surgency/extraversion, sociability and high-intensity pleasure) was associated with increased α-diversity in the microbiota of both sexes[102]. The association between temperament and microbiota has not yet been studied in animals, however, it has been shown that in stressed finishing cattle, calm temperament was associated with increased shedding of enterohemmorhagic *E. coli* O157:H7[99a]. While acute stress can be beneficial for animals, chronic stress negatively affects animal performance and well-being[175]. Increased blood cortisol concentrations are negatively associated with serum immunoglobulin concentrations and body weight gain in neonatal calves[176]. Weaning is associated with transition of calves from functioning as a monogastric to a ruminant. Prior to weaning calves are on a milk-based diet which is primarily digested in abomasum and intestine; after weaning calves are fed solid diet and must switch to ruminal fermentation[177]. While diet is a known factor that can change gut microbiota composition and metabolites rather quickly[178], the effect of sex classification and temperament on fecal microbiota and fecal or serum metabolites in cattle is unknown. The objective of this study was to 1) investigate the effect of weaning stress on the fecal microbiota as well as serum and fecal metabolites in Brahman calves, and 2) compare the fecal microbiota as well as serum and fecal metabolites between males (bulls) and females (heifers) as well as between calm and temperamental animals at weaning (d0) and 4 days post weaning (d4). The fecal

microbiota was also used to predict metabolic pathways affected by treatment. These predictions were compared with measured changes in metabolites.

4.3. Results

4.3.1. Temperament and serum cortisol.

According to temperament scores, 15% of animals were classified as temperamental (5 bulls, 7 heifers) 55% as intermediate (13 bulls, 30 heifers) and 30% as calm (15 bulls, 8 heifers).

The average serum cortisol concentration was 11.8±0.63 ng/mL, 18.0±0.68 ng/mL, and 34±0.69 ng/mL in calm, intermediate and temperamental animals respectively. In agreement with Pfeiffer *et al.*[99a], sex was associated with serum cortisol, where average serum cortisol concentration was higher in females (21.5±1.7 ng/mL) compared to males (14.6±2.0 ng/mL) ($P < 0.05$). We determined an association between temperament and increased serum cortisol concentrations ($P < 0.0001$) and gender ($P < 0.05$).

4.3.2. Dynamics of fecal microbiota, microbial metabolic functions, fecal and serum metabolites

4.3.2.1. Weaning and sex are associated with shifts in microbial beta diversity

The unweighted PCoA plot showed significant separation between the fecal samples from d0 and d4 after weaning (R = 0.301, $P < 0.001$) (Fig. 19A). The weighted PCoA plot showed significant separation between the fecal samples from d0 and d4 after weaning ($R = 0.1844$, $P < 0.001$) (Fig. 19B). The unweighted PCoA plot showed significant separation between the fecal samples from males and females at d4 (R =

0.1101, $P < 0.023$) (Fig. 19C), whereas weighted PcoA plot did not determine significant difference between males and females at d4 after weaning (Fig. 19D).

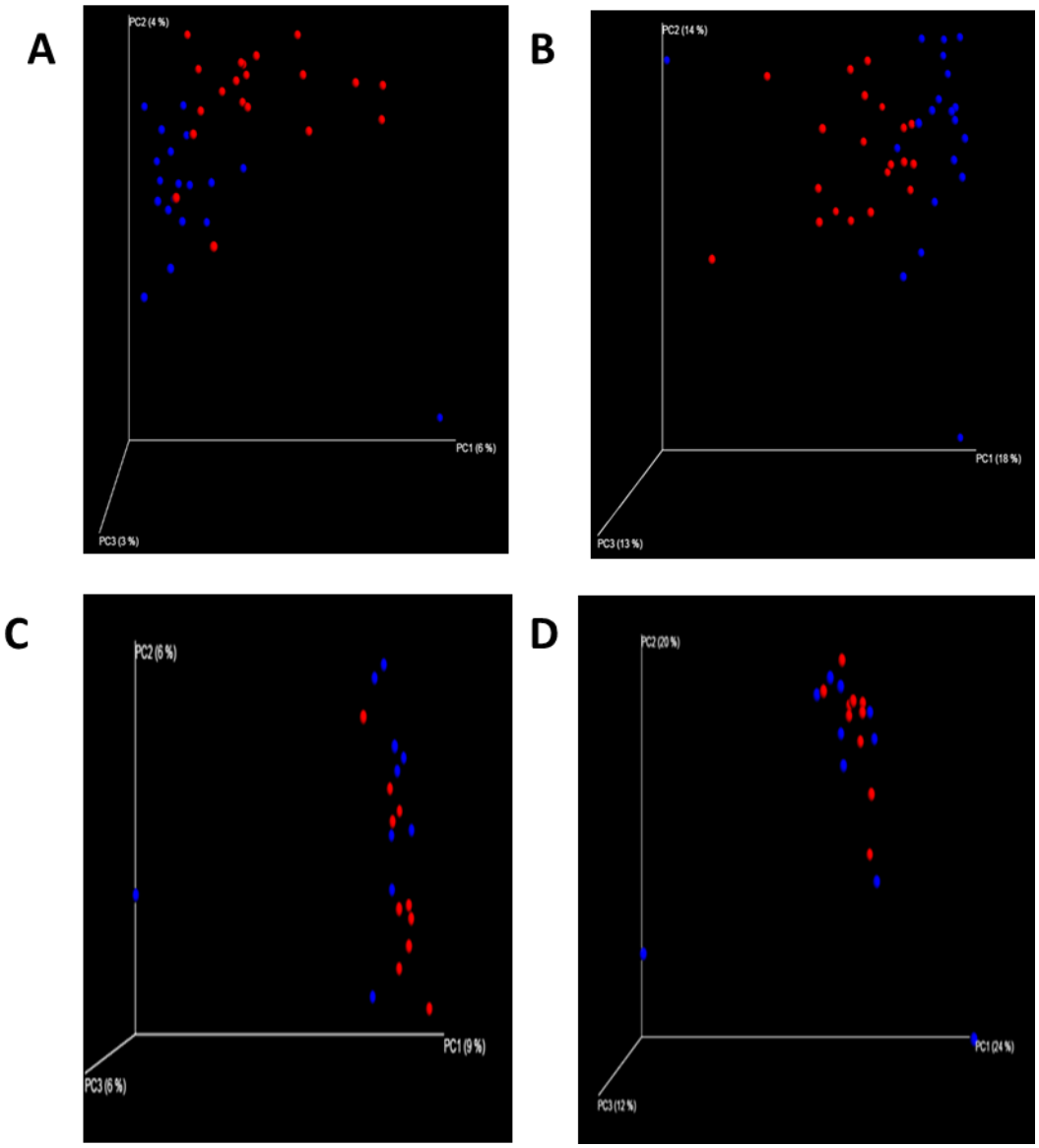

Figure 19: Microbial community structure differs between d0 and d4 after weaning and M and F at d4. Calves show significantly different beta diversity of gut microbiome at d0 (red) and d4 (blue) (A) unweighted unifrac (R=0.301, *P*<0.001), (B) weighted unifrac (R=0.1844, *P*<0.001). Males (blue) and females (red) exhibit different beta diversity at d4 (C) unweighted unifrac (R=0.1107, *P*=0.023), (D) weighted unifrac (R=0.0306, *P*=0.212).

These results indicate that overall microbial composition was different between sampling days as well as between males and females at d4 after weaning. Alpha diversity (chao1, observed species and Shannon index) did not differ between groups.

4.3.2.2. Weaning is associated with shifts in microbiota and predicted microbial metabolic functions

Univariate analysis (alpha value for the Wilcoxon Mann-Whitney test = 0.05, FDR = 0.1) showed that 50 microbial taxa were differentially abundant between d0 and d4 after weaning (Fig. 20). In particular, order Barnesiellaceae (class Bacteroidales) was enriched at d0, while BS11, RF16 orders of the same class were enriched at d4 after weaning. Genera *Lactobacillus*, *Streptococcu*s, *Coprobacillus*, *Sutterella*, PSB-M-3 as well as order RF39 were enriched at d0. Families Mogibacteriaceae, Veillonellaceae and Lachnospiraceae of order Clostridiales were enriched at d0 whereas families Caldicoprobacteraceae and Peptostreptococcaceae were enriched at d4 after weaning. Order SHA-98 was enriched at d4 after weaning. Genera *Campylobacter*, *Turicibacter* and *Treponema* were enriched at d4 after weaning.

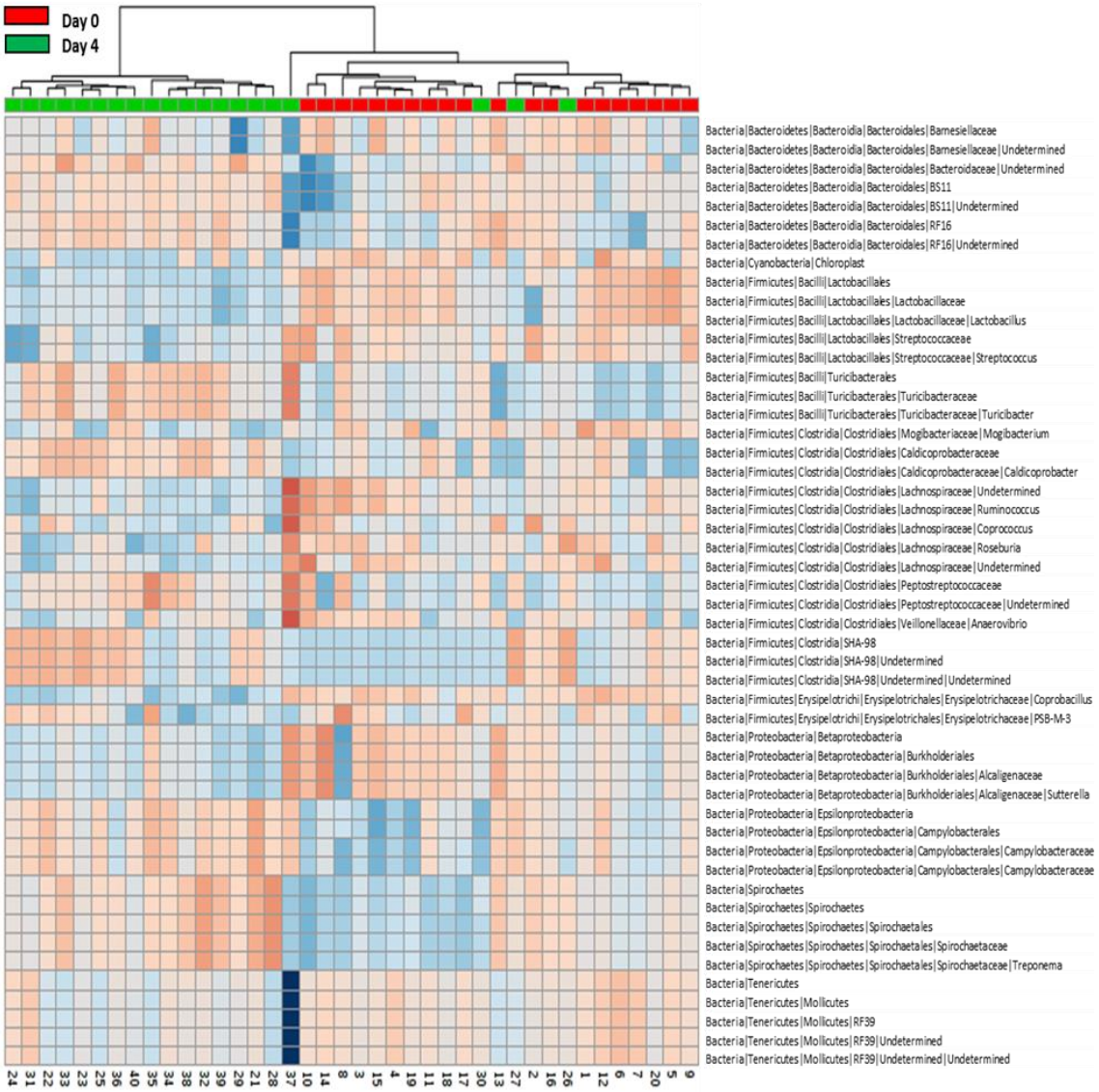

Figure 20: Heatmap showing bacterial taxa (97%-identity OTUs) altered (FDR-corrected $P < 0.1$) between sampling says and sexes at d4 after weaning. 71 taxa were differentially abundant between weaning and 4 days post-weaning. Samples are ordered by hierarchical clustering. Red and blue indicate increased and decreased levels, respectively.

Table 7: Predicted microbial metabolic pathways (3rd level of the KO hierarchy) differentially abundant (FDR-corrected *P* < 0.1) between weaning and 4 days post-weaning.

Metabolic pathway	Enriched
Environmental Information Processing|Membrane Transport|ABC transporters	d0
Genetic Information Processing|Transcription|Transcription machinery	d0
Human Diseases|Immune System Diseases|Primary immunodeficiency	d0
Human Diseases|Infectious Diseases|Bacterial invasion of epithelial cells	d0
Metabolism|Biosynthesis of Other Secondary Metabolites|Flavone and flavonol biosynthesis	d0
Metabolism|Carbohydrate Metabolism|Pentose and glucuronate interconversions	d0
Metabolism|Carbohydrate Metabolism|Pentose phosphate pathway	d0
Metabolism|Carbohydrate Metabolism|Starch and sucrose metabolism	d0
Metabolism|Lipid Metabolism|Glycerolipid metabolism	d0
Metabolism|Lipid Metabolism|Primary bile acid biosynthesis	d0
Metabolism|Lipid Metabolism|Secondary bile acid biosynthesis	d0
Metabolism|Metabolism of Other Amino Acids|D-Arginine and D-ornithine metabolism	d0
Metabolism|Metabolism of Other Amino Acids|Selenocompound metabolism	d0
Organismal Systems|Digestive System|Carbohydrate digestion and absorption	d0
Organismal Systems|Endocrine System|Adipocytokine signaling pathway	d0
Organismal Systems|Immune System|RIG-I-like receptor signaling pathway	d0
Cellular Processes|Transport and Catabolism|Lysosome	d4
Environmental Information Processing|Membrane Transport|Transporters	d4
Environmental Information Processing|Signaling Molecules and Interaction|Cellular antigens	d4
Environmental Information Processing|Signaling Molecules and Interaction|G protein-coupled receptors	d4
Genetic Information Processing|Folding, Sorting and Degradation|Protein processing in endoplasmic reticulum	d4
Genetic Information Processing|Transcription|Basal transcription factors	d4
Metabolism|Amino Acid Metabolism|Phenylalanine, tyrosine and tryptophan biosynthesis	d4
Metabolism|Amino Acid Metabolism|Tyrosine metabolism	d4
Metabolism|Biosynthesis of Other Secondary Metabolites|Isoquinoline alkaloid biosynthesis	d4
Metabolism|Biosynthesis of Other Secondary Metabolites|Novobiocin biosynthesis	d4
Metabolism|Biosynthesis of Other Secondary Metabolites|Tropane, piperidine and pyridine alkaloid biosynthesis	d4
Metabolism|Carbohydrate Metabolism|Citrate cycle (TCA cycle)	d4
Metabolism|Energy Metabolism|Carbon fixation pathways in prokaryotes	d4
Metabolism|Energy Metabolism|Oxidative phosphorylation	d4
Metabolism|Glycan Biosynthesis and Metabolism|Glycosphingolipid biosynthesis - ganglio series	d4
Metabolism|Glycan Biosynthesis and Metabolism|Peptidoglycan biosynthesis	d4
Metabolism|Lipid Metabolism|Fatty acid biosynthesis	d4
Metabolism|Lipid Metabolism|Fatty acid metabolism	d4
Metabolism|Lipid Metabolism|Lipid biosynthesis proteins	d4
Metabolism|Metabolism of Cofactors and Vitamins|Biotin metabolism	d4
Metabolism|Metabolism of Cofactors and Vitamins|Folate biosynthesis	d4
Metabolism|Metabolism of Terpenoids and Polyketides|Prenyltransferases	d4
Metabolism|Metabolism of Terpenoids and Polyketides|Terpenoid backbone biosynthesis	d4
Metabolism|Metabolism of Terpenoids and Polyketides|Zeatin biosynthesis	d4
Metabolism|Xenobiotics Biodegradation and Metabolism|Naphthalene degradation	d4
Metabolism|Xenobiotics Biodegradation and Metabolism|Polycyclic aromatic hydrocarbon degradation	d4
Organismal Systems|Endocrine System|PPAR signaling pathway	d4
Unclassified|Metabolism|Amino acid metabolism	d4

Based on PICRUST analysis, 39 and 263 metabolic functions (KEGG orthology (KO) database at hierarchy levels (2 and 3 respectively) were predicted based on OTUs matched GG 13.8 database. Forty-four metabolic functions predicted by analysis of the microbiota were differentially enriched between d0 and d4 after weaning at the KO level 3 (Table 7). Nineteen and 25 functions were enriched at d0 and d4 after weaning, respectively. In particular, pathways involved in membrane transport, signaling, virulence, bile acid biosynthesis and carbohydrate metabolism were enriched at d0. Pathways involved in tyrosine and tryptophan biosynthesis and metabolism, fatty acid metabolism, alkaloid metabolism, biosynthesis of antimicrobials (novobiocin), metabolism of cofactors and vitamins (folate and biotin), and PPAR signaling were enriched at d4 after weaning.

4.3.2.3. Weaning is associated with shifts in fecal and serum metabolites

A total of 199 identified and 194 unknown metabolites were identified in feces. 71 known fecal metabolites were differentially enriched between d0 and d4 after weaning (Fig. 21). Carbohydrates, lipids and organic acids were enriched at both days. Alkaloid xanthine was enriched at d0 while alkaloid nicotinamide was enriched at d4 after weaning. Nucleic acids uracil, thymine and guanine were enriched at d0 while adenosine was enriched at d4 after weaning. Peptides glutamic acid, glutamine, ethanolamine and asparagine were enriched at d4 after weaning.

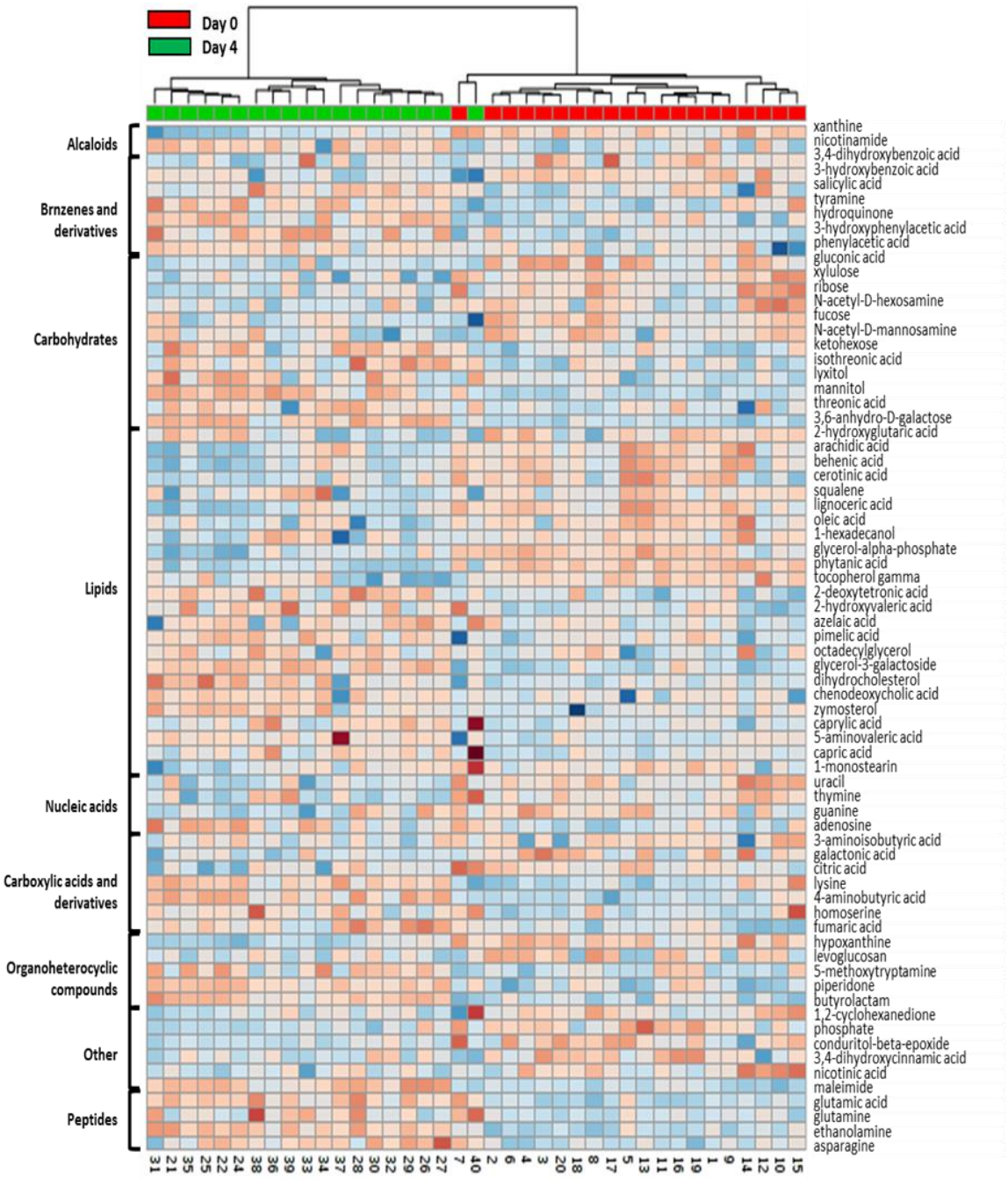

Figure 21: Heatmap showing fecal metabolites differentially abundant (FDR-corrected $P < 0.1$) between weaning and 4 days post-weaning. Metabolites are arranged by super pathway. Samples are ordered by hierarchical clustering. Red and blue indicate increased and decreased levels, respectively.

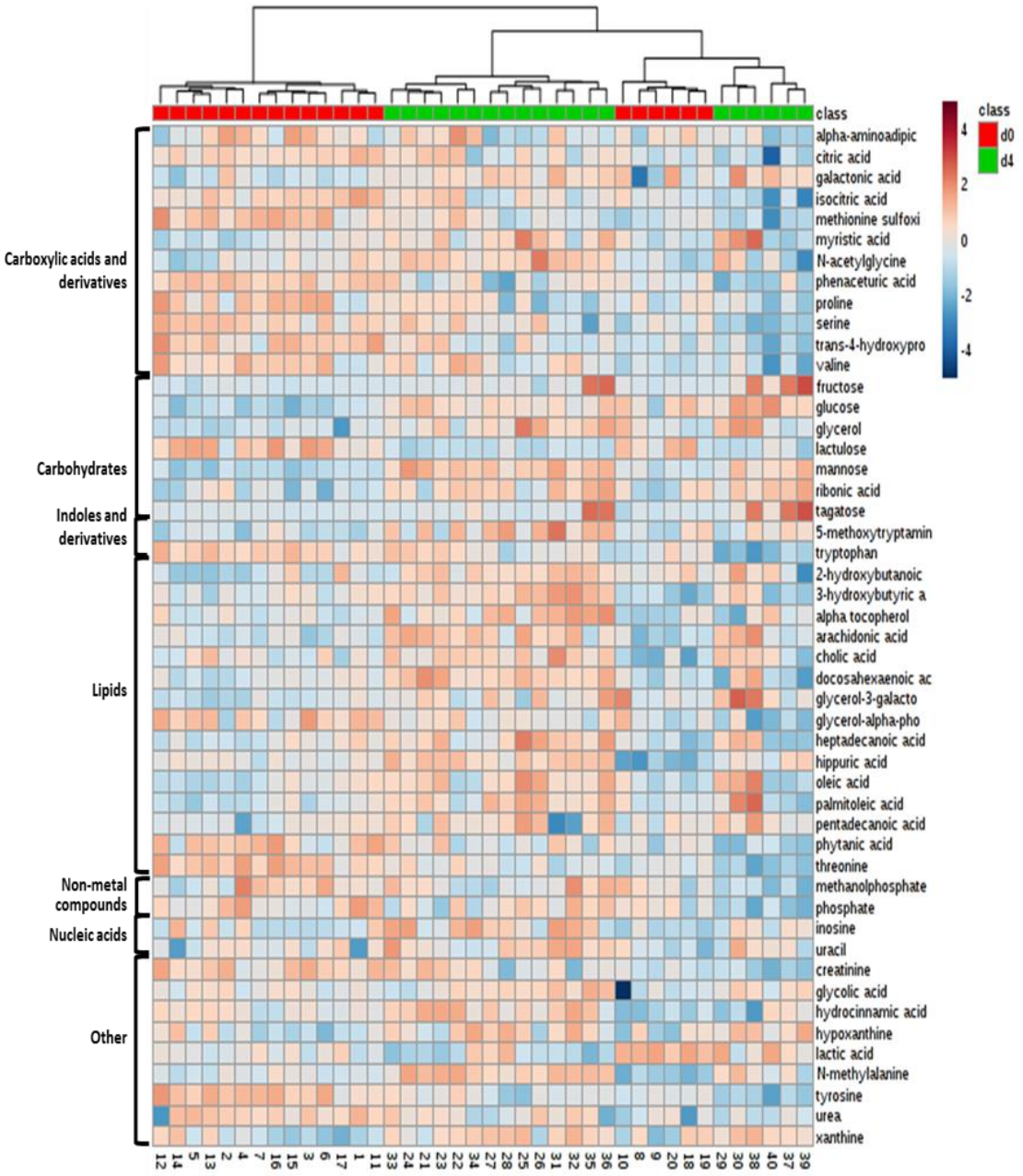

Figure 22: Heatmap showing serum metabolites differentially abundant (FDR-corrected $P < 0.1$) between weaning and 4 days post-weaning. Samples are ordered by hierarchical clustering. Red and blue indicate increased and decreased levels, respectively.

A total of 123 identified and 118 unknown metabolites were detected in serum. Forty-nine known serum metabolites were differentially enriched between d0 and d4 after weaning (Fig. 22).

The carbohydrate lactulose was enriched at d0, while fructose, glucose, glycerol, mannose, ribonic acid and tagatose were enriched at d4 after weaning. Consistent with predicted effects on metabolic pathways the indole derivative, tryptophan, was enriched at d0, while 5-methoxytryptamine was enriched at d4 after weaning. Both non-metal compounds methanolphosphate and phosphate were enriched at d0. Also consistent with predicted effects on metabolic pathways alkaloids xanthine and hypoxanthine were enriched at d4 after weaning.

4.3.2.4. Shifts in microbiota and fecal metabolites are associated with sex classification

Thirty-one microbial taxa were differentially enriched between males and females at d4 after weaning (Fig. 23). Kingdom Archaea (phylum Euryarchaeota, class Thermoplasmata, order E2, family Methanomassiliicoccaceae, genus *vadinCA11* as well as phylum Lentisphaerae (class Lentisphaeria, order Victivallales, family *Victivallaceae*, genus Undetermined). Phylum Verrucomicrobia (class Verruco-5, order WCHB1-41, family RFP12, genus Undetermined) was also enriched in females.

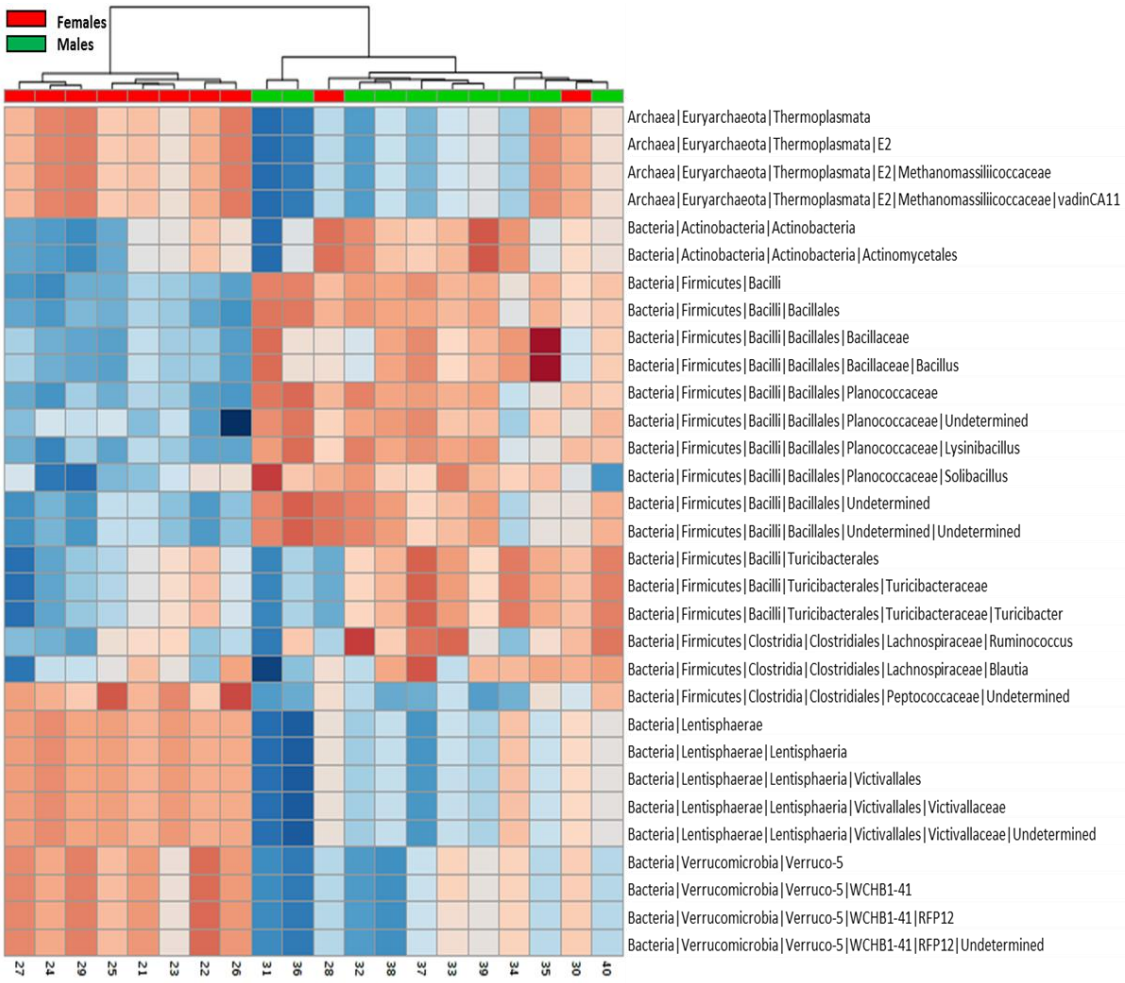

Figure 23: Heatmap showing bacterial taxa (97%-identity OTUs) altered (FDR-corrected *P* < 0.1) between sexes at d4. 31 taxa were differentially abundant between males and females at 4 days post-weaning. Samples are ordered by hierarchical clustering. Red and blue indicate increased and decreased levels, respectively.

Class Bacilli was enriched in males at d4 after weaning: order Bacillales, family Bacillaceae, class Bacillus as well as order Bacillales, family Planococcaceae, genera *Lysinibacillus*, *Solibacillus* and undetermined genus. Order Turicibacterales (family Turicibacteraceae, genus *Turicibacter* and undetermined genus) were also enriched in

males, as well as class Actinobacteria (family Actinomycetales) and family Lachnospiraceae (genera *Ruminococcus* and *Blautia*).

Nine identified fecal metabolites were differentially enriched between males and females at d4 (Table 8).

Table 8: Fecal metabolites differentially abundant (FDR-corrected *P* < 0.1) between males and females and between calm and temperamental calves at 4 days post-weaning.

Metabolite	Enriched
3,6-anhydro-D-galactose	Female_d4
4-aminobutyric acid	Female_d4
Adenine	Female_d4
Arachidonic acid	Male_d4
Fumaric acid	Female_d4
Hydroquinone	Female_d4
Maleimide	Female_d4
Phytanic acid	Male_d4
Tocopherol alpha	Male_d4
3-hydroxybenzoic acid	Calm_d4
Aspartic acid	Temperamental_d4
Caprylic acid	Temperamental_d4
Inosine	Temperamental_d4
Xanthine	Temperamental_d4

Maleimide, hydroquinone, 3,6-anhydro-D-galactose, adenine, 4-aminobutyric acid and fumaric acid were enriched in females, while arachidonic acid, phytanic acid and tocopherol alpha were enriched in males. No serum metabolites were differentially enriched between sexes.

4.3.2.5. Temperament is associated with shifts in microbiota, and metabolites in feces and serum

Seven microbial taxa were differentially abundant between calm and temperamental animals at d4 after weaning (Table 9). Family Erysipelotrichaceae, genus *PSB-M-3* and family Pirellulaceae were enriched in calm animals.

Table 9: Taxa (97%-identity OTUs) differentially abundant (FDR-corrected *P* < 0.1) between calm and temperamental calves at 4 days post-weaning.

Microbial taxa	Enriched
Bacteria|Elusimicrobia|Elusimicrobia|Elusimicrobiales|Elusimicrobiaceae	Calm
Bacteria|Firmicutes|Erysipelotrichi|Erysipelotrichales|Erysipelotrichaceae|PSB-M-3	Calm
Bacteria|Planctomycetes	Calm
Bacteria|Planctomycetes|Planctomycetia	Calm
Bacteria|Planctomycetes|Planctomycetia|Pirellulales	Calm
Bacteria|Planctomycetes|Planctomycetia|Pirellulales|Pirellulaceae	Calm
Bacteria|Planctomycetes|Planctomycetia|Pirellulales|Pirellulaceae|Undetermined	Calm

Five fecal metabolites were differentially enriched between calm and temperamental animals at d4 after weaning (Table 10). 3-hydroxybenzoic acid was enriched in calm calves, while aspartic acid, inosine, xanthine and caprylic acid were enriched in temperamental calves.

Ten serum metabolites were differentially enriched between calm and temperamental calves at d0 (Table 10).

Table 10: Serum metabolites differentially enriched between calm and temperamental animals at d0, d4 and males at d4. FDR<0.1.

Metabolite	Enriched
Calm_d0 vs Temperametal_d0	
Aspartic acid	Calm
Hippuric acid	Calm
Hydrocinnamic acid	Calm
Phenaceturic acid	Calm
2-hydroxybutanoic acid	Temperamental
Citramalic acid	Temperamental
Glycerol-3-galactoside	Temperamental
Lactic acid	Temperamental
Malic acid	Temperamental
Sorbitol	Temperamental
Calm_d4 vs Temperamental_d4	
Tryptophan	Calm
Citrulline	Calm
Glycerol-3-galactoside	Temperamental
Males_d4: Calm vs Temperametal	
Hydroxylamine	Calm
Inosine	Calm
Isoleucine	Calm
Glucose	Temperamental
Trehalose	Temperamental

Aspartic acid, hippuric acid, hydrocinnamic acid and phenaceturic acid were enriched in calm calves. However, 2-hydroxybutanoic acid, citramalic acid, glycerol-3-galactoside, lactic acid, malic acid and sorbitol were enriched in temperamental calves at weaning. Three serum metabolites were differentially enriched between calm and temperamental calves at d4 after weaning (Table 10). Tryptophan and citrulline were enriched in calm animals, while glycerol-3-galactoside was enriched in temperamental

animals. Five serum metabolites were differentially enriched between calm and temperamental males at d4 after weaning (Table 10). The sugars glucose and trehalose were enriched in temperamental males, while hydroxylamine, inosine and isoleucine were enriched in calm males.

4.3.2.6. Weaning, sex and temperament are associated with shifts in neuroactive fecal and serum metabolites

In feces, 3-aminoisobutyric acid, xanthine and hypoxanthine were enriched at weaning (Table 11). At d4 after weaning were enriched 5-aminovaleric acid, 5-methoxytryptamine, adenosine, capric acid, glutamic acid, tyramine and 4-aminobutyric acid (GABA). GABA was also enriched in females at d4 after weaning. Arachidonic acid was enriched in males at d4. Additionally, xanthine, aspartic acid and inosine were enriched in temperamental animals at d4 post-weaning. In serum at weaning were enriched alpha-aminoadipic acid, lactic acid, lactulose, serine, tryptophan, tyrosine and urea (Table 10). At d4 after weaning were enriched 3-hydroxybutyric acid, 5-methoxytryptamine, arachidonic acid, docosahexaenoic acid, hydrocinnamic acid, hypoxanthine, inosine, mystiric acid, uracil and xanthine. In calm animals at weaning were enriched aspartic acid and hydrocinnamic acid. In calm animals at d4 after weaning were enriched tryptophan and citrulline. Lactic acid was enriched in temperamental animals at d0. Inosine was enriched in calm males at d4 after weaning, while trehalose was enriched in temperamental males at d4.

Table 11: Differentially abundant (FDR-corrected *P* < 0.1) fecal and serum metabolites which have neuroactive function.

Metabolite	Enriched	Function
Neuroactive metabolites enriched in feces		
3-aminoisobutyric acid	d0	Neuroactive molecule
3-aminoisobutyric acid	d4	Antagonist for GABAB receptors
4-aminobutyric acid (GABA)	d4, Females_d4	Inhibitory neurotransmitter
5-methoxytryptamine	d4	Serotonin derivative, neurotransmitter
Adenosine	d4	Neurotransmitter
Aspartic acid	Temperamental_d4	Neurotransmitter, stimulates NMDA receptors
Capric acid	d4	AMPA receptor antagonist
Glutamic acid	d4	Excitatory neurotransmitter, acts on NMDA and AMPA receptors
Hypoxanthine	d0	CNS stimulant, adenosine antagonist
Inosine	Temperamental_d4	Neuroprotector, improves axonal rewiring
Tyramine	d4	Neurotransmitter, acts on TAAR1 receptor
Xanthine	d0, Temperamental_d4	CNS stimulant, adenosine antagonist
Neuroactive metabolites enriched in serum		
3-hydroxybutyric acid	d4	Enkephalinase inhibitor. Increases brain enkephalin concentration
5-methoxytryptamine	d4	Enkephalinase inhibitor. Increases brain enkephalin concentration
Alpha-aminoadipic acid	d0	Neuroprotective properties
Arachidonic acid	d4	CNS stimulant, adenosine antagonist
Aspartic acid	Calm_d0	Neuroprotective functions
Aitrulline	Calm_d4	Neuroprotective functions
Docosahexaenoic acid	d4	Neuroprotector, improves axonal rewiring
Hydrocinnamic acid	Calm_d0	Precursor of dopamine, Epi and NE
Hydrocinnamic acid	d4	Neuroprotector, improves axonal rewiring
Hypoxanthine	d4	Signals to neurons to release noradrenaline
Inosine	Calm_Males_d4	Neurotoxic
Inosine	d4	Enhances neuroplasticity, signal to neurons to release noradrenaline
Lactic acid	d0	Serotonin derivative, neurotransmitter
Lactic acid	Temperamental_d0	Neuroregenerative, stimulates serotonine release
Lactulose	d0	Gliotoxic
Myristic acid	d4	Enhances neuroplasticity
Serine	d0	Brain phospholipid, released in response to inflammation
Trehalose	Temperamental_Males_d4	CNS stimulant, adenosine antagonist
Tryptophan	Calm_d4	Neuroprotective
Tryptophan	d0	Neurotransmitter, stimulates NMDA receptors
Tyrosine	d0	Neurogenesis
Uracil	d4	Anxiolytic
Urea	d0	Structural component of neuronal membranes.
Xanthine	d4	Involved in glia-synapse interactions

4.3.2.7. Weaning and temperament are associated with shifts in metabolism and catabolism in serum

Several metabolites associated with lipolysis were differentially enriched in serum. Glycerol, glycerol-3-galactoside, oleic acid and palmitoleic acid were enriched at d4 after weaning. Additionally, glycerol-3-galactoside was enriched in temperamental animals at weaning and d4 after weaning. Glycolysis-associated metabolites were differentially enriched between sampling days and temperaments. We determined that fructose, glucose, galactonic acid and tagatose were enriched at d4 after weaning. Glucose and trehalose were enriched in temperamental animals at d4 after weaning while malic acid was enriched in temperamental animals at weaning. Additionally, several markers of glycogenesis/glyconeogenesis, such as citric acid, lactic acid, lactulose, methionine sulfoxide, phenaceturic acid, proline, serine, threonine, tryptophan, tyrosine and valine were enriched at weaning.

4.3.2.8. Correlation between significantly enriched fecal and serum metabolites

At weaning lactic acid in serum negatively correlated with fumaric acid and maleimide in feces. At d4 3,6-anhydro-D-galactose in feces negatively correlated with fructose and tagatose in serum. Additionally, in serum at weaning lactic acid negatively correlated with citric acid, phenaceturic acid and hippuric acid.

4.4. Discussion

4.4.1. Temperament, sex and cortisol

In agreement with previous research[99a, 176] excitable temperament was associated with increased serum cortisol concentrations in Branham calves at weaning. Females had

higher average serum cortisol concentration compared to males and the association between serum cortisol and temperament was greater in females. Cortisol has been negatively associated with feed intake, metabolism, nutrient partitioning and reproduction in cattle[179]. For example, temperamental heifers reach puberty later and have reduced pregnancy rates when compared to calm heifers[179]. On the other hand, calm and intermediate animals are more susceptible to endotoxin challenge and have more severe response to immunological stress compared to temperamental animals[180]. This suggests that temperament can have both positive and negative effects on animal physiology. Association of temperament and sex was noticed at cow-calf operations, where male calves were easier to handle compared to females[181]. Similarly, Angus beef heifers tend to have greater basal serum cortisol concentrations compared to bulls[182]. Alternatively, testosterone was associated with greater docility in cattle[183] which may partially explain sexual dimorphism in the basal serum cortisol concentrations in the present study.

4.4.2. Microbiota

4.4.2.1. Day

In agreement with previous studies[184] , the major bacterial phyla present in feces were Bacteroidetes (47.3%), Firmicutes (43.3%) Proteobacteria (1.5%), Tenericutes (3.2%) and Verrucomicrobia (2.6%). While we determined that there is a significant effect of weaning on beta diversity of the fecal microbiota, there was no change in the alpha diversity. This indicates that while differences in species composition among samples were changing between sampling days (beta diversity), the alpha diversity,

represented by richness (number of species present in a sample) and evenness (relative abundance of species that make up the richness in that area), remained the same at d0 and d4 after weaning. The dissimilarity between d0 and d4 after weaning was more pronounced using unweighted UniFrac diversity metrics (R=0.301), which uses the presence and absence of OTUs and phylogeny compared to weighted UniFrac (R=0.1844), which uses the abundance information of OTUs and phylogeny. This suggests that less abundant OTUs contributed to the difference in beta diversity between d0 and d4 after weaning. At weaning animals experience acute stress, whereas at 4 days post-weaning calves are suffering chronic stress. Additionally, we determined a difference in beta diversity between males and females at d4. It has been shown that females are more stress-responsive than males[185 185a]. Use of unweighted Unifrac metrics yielded larger dissimilarity between sexes (unweighted R=0.1107, weighted R=0.0306), indicating contribution of low abundant taxa to diversity between sexes. Lack of difference between sexes at weaning may be due to low sex-dependent microbiota separation before puberty[186]. Weaning stress and associated change in diet may amplify difference between sexes, which may explain the difference in beta diversity between males and females at d4 after weaning.

Weaning is a complex process which involves change of diet and induction of stress from maternal separation and change of housing. Diet and stress of various etiologies have been shown to have a significant effect on fecal microbiota[96, 178, 187]. In agreement with previous studies which sampled fecal and rumen microbiota of weaning calves[187a, 188], we determined that phylum Spirochaetes, as well as genera *Treponema*

and *Coprococcus* were significantly increased in feces post-weaning while genera *Lactobacillus* and *Streptococcus* were enriched at weaning. However, contrary to published results[188], class Mollicutes as well as genera *Roseburi*a, *Ruminococcus* and *Anaevibrio* were enriched at weaning. Additionally, our data supports previous research on the fecal microbiota in weaned pigs[189], which showed that family Spirochaetaceae was enriched in feces after weaning. We also found that families Lactobacillaceae and Streptococcaceae were enriched after weaning, agreeing with Dickved et al[187a] and disagreeing with Frese et al[189]. The discrepancies between studies may be attributed to host animal species, as well as the difference in diet and weaning age. For example, a previous study, which sampled calves on day 36 (pre-weaning) and day 54 (post-weaning) of life, have found that abundance of *Ruminococcus*, was increased after weaning[188]. We sampled calves at weaning (4-6 months of age), and 4 days after weaning (post-weaning) and determined that *Ruminococcus* decreased post-weaning. Interestingly, it has been shown that weaning strategy does not affect fecal microbiota in calves[188] and that in contrast to rumen microbiota, fecal microbiota had a greater richness and evenness post-weaning[189 188]. Our findings indicate that weaning has a significant effect on fecal microbial composition in livestock.

4.4.2.2. Sex

Sex-related differences in commensal microbiota composition have been well established in mice[190]. A previous study has investigated sex differences in gut microbiota composition in 89 different inbred strains of mice after puberty and discovered significant differences in beta-diversity and average relative abundances at

various taxa levels between males and females. In particular, genera *Alloceculum*, *Anaeroplasma* and *Erwinia* were enriched in males, while *Dorea, SMB53, Ruminococcus* and *Coprococcus* were enriched in females[186]. In disagreement with the above study, we determined that *Ruminococcus* increased in males at d4 after weaning. Another study determined clear differences in fecal microbial composition between adult male and female mice, where in agreement with our results, Peptococcaceae was enriched in females[191]. The effect of sex on microbiota is seen in mammals after puberty [190, 192], however, studies done in early life have determined only zero to moderate effect of sex on gut microbiota[187d, 193].

4.4.2.3. Temperament

It was shown that nesting stress in rats increases cortisol concentrations and decreases fecal microbiota diversity, but also increases relative abundance of gram positive cocci (*Enterococcus, Streptococcus, Peptococcus, Aerococcus, Jeotgalicoccus*, and *Facklamia*) at weaning[187d]. While stress induces microbial changes, microbiota shapes HPA response in mammals at an early stage of life[92]. Lack of microbiota results in elevated stress-induced plasma ACTH and adrenal cortical steroid secretion which can be decreased by administration of probiotic *B. infantis*, but increases even more after administration of pathogenic *E. coli*. Additionally, the exaggerated HPA response in GF mice was decreased by transfer of feces from SPF mice, but only at early life stage (9 weeks of age) and not later (17 weeks of age)[92]. Excitable temperament is associated with increased basal serum cortisol concentrations as well-as elevated stress-induced cortisol concentrations[99]. A recent study determined an association between

temperament and gut microbiota in toddlers, where greater surgency/extraversion was associated with greater phylogenetic diversity in both genders [102]. A study in Siberian hamsters has shown that antibiotics differentially affect the gut microbiota and aggressive behavior across the sexes [49]. However, there was a lack of association between gut microbiota and psychiatric measures (anxiety, depression, eating-related thoughts and behaviors, stress, or personality) in healthy human females [50]. Although, transportation stress has been shown to increase *E. coli* O157:H7 shedding in calm cattle[99a], there is very limited information on the association between temperament and gut microbiota. In this study we determined that the Elusimicrobiaceae family, genus *PSB-M-3* and undetermined genus of the Pirellulaceae family were enriched in feces of calm calves 4 days post-weaning. Elusimicrobiaceae are gram negative obligate anaerobes with purely fermentative metabolism[194] and Pirellulaceae is a member of the rumen bacterial community in cattle[195]. These data suggest an association between temperament types and gut microbiota in cattle; however, the nature of this association and importance of certain bacterial taxa requires further investigation.

4.4.3. Metabolites

4.4.3.1. Day

Weaning stress is associated with significant shifts in metabolism[196]. An array of metabolites was differentially enriched in feces and serum of pre-weaned and weaned calves in the present study. In particular, the serotonin derivative, neurotransmitter 5-methoxytryptamine[197], and an intermediate in galactose and glycerolipid metabolism, glycerol-3-galactoside hypoxanthine were enriched in serum and feces at d4 after

weaning. An adenosine antagonist and CNS stimulant hypoxanthine, glycerol-alpha-phosphate, phosphate and phytanic acid were enriched in serum and feces at weaning. Citric acid, which have been associated with increased rumen metabolism[198] was enriched in serum and feces at weaning. Additionally, arachidonic and docosahexaenoic acid, which make up to 20% of fatty acids in the mammalian brain and must be obtained from dietary sources[199] were enriched in serum at d4 after weaning. Thus, present study suggests that weaning is associated with significant shifts in metabolism in the cattle. Microbiota is involved in regulation of blood metabolites. For example, compared to GF mice, conventionally raised mice had increased microbial metabolite hydrocinnamic acid, energy metabolites citric and malic acid, glucose and proline in serum. However, serum urea and the amino acids valine and tryptophan were decreased compared to GF mice[200]. We determined that serum hydrocinnamic acid was increased at d4 after weaning and in calm animals at weaning, citric acid was increased at weaning and malic acid was increased in temperamental animals at weaning. Glucose was enriched at d4 after weaning and in temperamental animals post-weaning. Proline, urea, valine and tryptophan were enriched at weaning. These data suggest that weaning-, and temperament-associated differences in concentrations of serum metabolites are partially determined by differences in microbial composition.

4.4.3.2. Sex

Several members of the gut microbiota, such as *L. plantarum*, *L. brevis*, *B. adolescentis*, *B. angulatum* and *B. dentium* can produce GABA from its precursor monosodium glutamate.

The *gadB* and *gadC* genes were identified in the following genera of bacteria: Bacteroidetes (Bacteroides, Parabacteroides, Alistipes, Odoribacter, Prevotella), Proteobacterium (Esherichia), Firmicutes (Enterococcus), Actinobacteria (Bifidobacterium). The present study determined that an inhibitory neurotransmitter GABA was enriched in the feces of females at d4 after weaning and polyunsaturated fatty acid, arachidonic acid, was enriched in feces in males at d4 after weaning. A human study revealed that of 507 identified serum metabolites, one-third was significantly different between males and females. Pathway analysis revealed strong differences in steroid metabolism, fatty acids and further lipids, fraction of amino acids, oxidative phosphorylation, purine metabolism and gamma-glutamyl dipeptides[201]. In rats, sex differences in ascorbic acid and pantothenic acid, but not in steroid metabolites, were prominent in serum[202]. The basal concentrations of glucocorticoid metabolites are higher in hamster males[203] and male rats comparing to females[204]. However, virtually no information exists on sex-induced difference in fecal metabolites in pre-pubertal animals.

The long-chain omega-3 polyunsaturated fatty acid docosahexaenoic acid has been shown to have a beneficial effect on reducing anxiety and depression. Docosahexaenoic acid supplementation has been reported to have a significant effect on microbial profiles in males but not in females and to produce beneficial effects on anxiety and depressive-like behaviors in a sex-specific manner[192]. We observed higher concentrations of docosahexaenoic acid at weaning than post-weaning; however, we did

not observe differences in docosahexaenoic acid concentration between sexes or temperaments.

4.4.3.3. Temperament

Neuroendocrine response to stress activates the hypothalamic–pituitary axis (HPA) which results in secretion of steroid hormones from the adrenal gland that increases the mobilization of energy by increased glycogenolysis, glycolysis, and proteolysis[99b, 100]. In our study we determined that lipolysis metabolites in serum, such as glycerol, glycerol-3 galactoside, oleic acid and palmitoleic acid were enriched post-weaning and in temperamental animals at weaning and post-weaning (glycerol-3-galactoside). Additionally, glycolysis sugars fructose, glucose, galactonic acid and tagatose were enriched post-weaning, while glycolysis metabolites glucose, trehalose, malic and lactic acid were enriched in temperamental animals. On the other hand, glycogenesis/glyconeogenesis metabolites such as amino acids proline, serine, methionine, threonine, tryptophan, tyrosine and valine were enriched at weaning.

We determined that glycerol-3-galactoside, an intermediate in galactose and glycerolipid metabolism, was enriched in temperamental animals in serum at d0 and d4 after weaning. Sugars, glucose and trehalose, were more abundant in serum of temperamental males at d4 after weaning while purine nucleoside inosine, amino acid isoleucine and inorganic compound hydroxylamine were more abundant in calm males at d4 after weaning.

Temperamental animals (males and females) had more abundant sugars in serum: glycerol-3-galactoside (intermediate in galactose metabolism) (d0 and d4 after weaning),

sorbitol (d0), glucose and trehalose (temperamental males d4 after weaning). Additionally, we determined that 2-hydroxybutanoic acid was increased in temperamental calves at weaning, and has been associated with insulin resistance and glucose tolerance[205]. A previous study has shown that during glucose tolerance testing, temperamental steers had greater serum glucose, yet decreased serum insulin compared to calm cattle, indicating increased insulin resistance[206]. We determined that temperamental animals had higher serum lactate concentrations at weaning. It has been shown that humans with impaired glucose tolerance had significantly higher mean lactate concentrations compared to those with normal glucose tolerance[207]. Research suggests that an excitable temperament is associated with decreased growth rate, carcass quality and increased shear force[208] as well as increased blood lactate[209].

Our study found that aspartic acid was increased in serum of calm calves at weaning. This was consistent with previous work which has shown that aspartic acid was decreased in the prefrontal cortex of fearful/neophobic-alert cows and increased in uninterested-calm and outgoing/neophilic-alert cows[210]. Interestingly, a decreased concentration of GABA but also of glutamate and aspartate have been reported in the prefrontal cortex and plasma of humans suffering from a melancholic major depressive disorder[211] thus indicating a behavioral effect of these amino acids. It has been shown that calm sheep had lover plasma insulin and leptin concentrations compared to temperamental sheep[212].

The amino acids arginine and citrulline act as nitric oxide precursors. The results of a previous study demonstrate a reduction in plasma lactate after citrulline

supplementation[213]. In our study lactate was increased in serum of temperamental calves at weaning, while citrulline was higher in calm calves at d4 after weaning. Additional studies are needed to determine if there is increased nitric oxide production in calm cattle as compared to temperamental cattle.

These data suggest that temperament is associated with differentially enriched neuroactive metabolites and energy metabolism. Additionally, weaning stress is also associated with differentially enriched neuroactive metabolites, however, energy metabolism is also affected.

4.5. Conclusions

It is important to note that the limitations in 16S sequencing allowed us to determine microbial taxa only up to genus level, leaving out information about species prevalence. Additionally, active gut microbiota (live cells) may differ from the total microbiota[214] and fecal samples do not fully represent the microbial and metabolic composition of the bovine gut[184a]. Despite these limitations this study determined that weaning is associated with dramatic shifts in fecal microbiota as well as fecal and serum metabolites in Brahman calves. To our surprise, gender of the animals which have not yet not reached puberty was associated with shifts in fecal microbial composition and fecal metabolites after weaning. Additionally, this study has shown for the first time that temperament of the animal is associated with differentially abundant microbiota as well as fecal and serum metabolites. These metabolites may have neuroactive function as well as being involved in energy metabolism.

4.6. Materials and methods

4.6.1. Animal selection and housing

All experimental procedures were approved by IACUC#2013-015A. Brahman calves aged 6 to 7 months comprised of 45 heifers and 33 bulls were selected for this study. Fecal and blood samples were taken at weaning (d0) and 4 days post-weaning (d4). Prior to weaning calves were housed in the pasture and were nursing and fed Coastal Bermudagrass. After weaning calves were housed in pen and fed in groups. Meals consisted of Coastal Bermudagrass and 4 lb/head/day of ground corn and soybean meal in proportion 3:1.

4.6.2. Assessment of temperament

Temperament of each calf was evaluated using pen score and exit velocity as previously described[99a, 100]. Exit velocity was determined by the rate at which an animal exited the working chute and travelled a fixed distance of 1.83 m, measured by two infrared sensors connected to an electronic timing unit. Pen scores were based on the individual animal's response to the assessors as they approached the animal. Pen scores and chute scores were based on a 1–5 scale, where 1 was a completely calm animal, and 5 was an extremely excited animal[215].

4.6.3. Blood sample collection

Blood was collected via jugular venipuncture into vacutainer tubes without an anti-clotting reagent. Samples were allowed to coagulate on ice followed by centrifugation at 1600 x g for 30 min. Serum was aspirated and stored at -20 ºC.

4.6.4. Rectal sampling

Fecal samples were collected via rectal grab while the animal was restrained in a squeeze chute. Samples were placed on ice immediately after collection and transported to the lab in insulated coolers on ice and processed within 24 h after collection.

4.6.5. Serum cortisol concentration

Serum cortisol concentration was determined by radioimmunoassay as previously described[216]. Briefly, rabbit anti-cortisol antiserum was diluted 1:2500. Standards were made by serial dilution according to the manufacturer's protocol. Sample cortisol concentrations were calculated using Assay Zap software (Biosoft, Cambridge, UK) and counts per minute which were obtained from a scintillation spectrophotometric beta-counter (Beckham Coulter LS 6500).

4.6.6. Microbiome assessment

Bacterial DNA was extracted using the PowerFecal kit (MoBio, Carlsbad, CA). Illumina sequencing of the V4-V6 region (*E. coli* position 530-1100, 530F GTGCCAGCMGCNGCGG, 1100R GGGTTNCGNTCGTTR) of the 16S rRNA was performed on a MiSeq (MR DNA, Shallowater, TX). The mapping file was checked for errors, and raw sequences (5229019) were de-multiplexed. Quality parameters were: Q3, minimum number of consecutive high quality base calls as a fraction of the input read length = 0.75. The maximum number of consecutive low-quality base calls allowing before truncating a read = 3. Maximum number of N characters allowed in a sequence = 0. After filtering 4773543 reads remained. USEARCH 6.1 was used to remove chimera sequences (662569), therefore 4110974 reads remained. Remaining

reads were used for open-reference OTU picking, where reads were clustered against GreenGenes 13.8 reference database and any reads which did not hit the reference sequence collection were subsequently clustered de novo. OTUs were defined as sequences with at least 97% similarity to GreenGenes 13.8 reference database. De-novo OTUs were removed and only OTUs matching GreenGene 13.8 (3724743) were used for phylotypic investigation of communities by reconstruction of unobserved states (PICRUST) analysis. The analysis involved normalization of OTUs by copy number, prediction of function and further categorization by function. The resulting metagenomics data were analyzed using Metaboanalyst 3.0 software.

4.6.7. Metabolite assessment

Fecal and serum samples for metabolite assays were thawed on ice, aliquoted and further stored in -80°C prior to extraction. Fecal samples were extracted following the published protocol[217], NIH West Coast Metabolomics Center, CA). Briefly, sample aliquots were extracted by degassed acetonitrile:isopropanol:water (3:3:2, v/v/v) at –20°C, centrifuged, decanted with subsequent evaporation of the solvent, extracted with acetonitrile:water (1:1) and desiccated. Internal standards C08-C30 FAMEs were added to the sample which was further extracted by methoxyamine hydrochloride in pyridine and by N-methyl-N-trimethylsilyltrifluoroacetamide.

Data were acquired and processed following a published procedure[218], NIH West Coast Metabolomics Center, CA). Briefly, ChromaTOF vs.2.32 software was used for data processing and BinBase algorithm was implemented for metabolite annotation.

Metaboanalyst 3.0 software (http://www.metaboanalyst.ca/). Data missing variables in 50% or more of samples were removed. Data were then normalized by sum, log-transformed, mean centered and divided by the standard deviation of each variable.

4.6.8. Statistical analyses

Nominal logistic regression modeling was used to test the associations between temperament and cortisol concentration or gender (JMP, SAS Institute). A significance value of $P < 0.05$ was selected for all statistical tests. Odds ratios (OR) were calculated for the temperamental and intermediate animals; the calm temperament served as a baseline. Nominal logistic regression with a single factor was used to determine individual effect of cortisol concentrations and gender on temperament. A two-tailed *t*-test was used to compare cortisol concentrations between genders. Differences in microbiota composition between sampling days, sex and temperament were assessed by unweighted and weighted UniFrac distance metrics and plotted on principal coordinate analysis (PCoA) plots. The Analysis of similarities (ANOSIM) function was used on UniFrac distance matrices to determine the statistical difference of microbiota between groups ($P<0.001$). Univariate analysis (alpha value for the Wilcoxon Mann-Whitney test = 0.05, FDR = 0.1) was used to determine differentially abundant microbial taxa, metabolic pathways and metabolites. Summary information of the representation (relative abundance) of taxonomic groups within each sample (summarize_taxa.py) – OTUs were correlated with fecal metabolite relative abundance). Correlations were performed in R using "rcorr" function of Hmisc. Relationships with Pearson's correlation coefficient >0.9 and <-0.9 were used for further analysis.

5. EFFECT OF ADRENERGIC AGONISTS ON MICROBIAL GROWTH, MOTILITY AND HORIZONTAL GENE TRANSFER

5.1. Summary

The stress hormone cortisol has been shown to induce growth of gram-negative bacteria while catecholamines (epinephrine and norepinephrine, 3,4- dihydroxymandelic acid (DHMA)) have been shown to increase bacterial motility. Adrenergic agonists such as ractopamine, zilpaterol, salbutamol, and terbutaline are structurally similar to NE and Epi and are able to bind to host adrenergic receptors. Possible participation of synthetic AAs in bacterial communication raises concerns regarding their use in animals and humans, although their effect on bacterial virulence and HGT needs to be further evaluated. The goal of this study is to determine whether DHMA and adrenergic agonists (AA) increase 1) growth and motility of *Salmonella enterica* serotype Typhimurium and 2) horizontal gene transfer (HGT) of plasmid carrying antimicrobial resistance genes between *S.* Typhimurium and commensal *Escherichia coli in vitro* NE, DHMA and AAs did not affect growth of *S.* Typhimurium in LB broth. We determined that ractopamine (50 and 1000 µM) decreased motility of *S.* Typhimurium in 0.3% agar whereas DHMA (0.5 and 5 µM) and NE (50 µM) increased motility. Terbutaline and salbutamol had no effect on motility. NE and ractopamine had no effect on HGT whereas DHMA (150 µM) had a tendency (P=0.078) to decrease HGT at 4h post-inoculation. These data indicate the importance of catecholamines and AAs in regulation of *S.* Typhimurium motility and their potential effect on HGT.

5.2. Introduction

The average, healthy mammalian gut hosts trillions of bacterial organisms, some of which can utilize horizontal gene transfer (HGT) via transformation, conjugation and transduction to facilitate adaptation changes. HGT is most efficient among closely related bacterial species, although inter-phyla and inter-kingdom HGT has been described[219]. Epinephrine (Epi) and norepinephrine (NE) are key catecholamine hormones produced by the central nervous system (CNS) and adrenal medulla. NE is also produced by the enteric nervous system where it acts as neurotransmitter. Both Epi and NE are present in the mammalian gut and participate in host signaling via binding to adrenergic receptors[13b, 76]. Microbes communicate with each other through various compounds. The bacterial autoinducer AI-3 is produced by microbiota but can be used by enteropathogenic bacteria, such as *E. coli* and *Salmonella*, to regulate growth, motility and virulence[220] as well as *Salmonella* survival in macrophages[36b]. Several compounds, including host Epi and NE, mimic the effect of AI-3[221]. The relationship between catecholamines and bacteria is complex with each affecting the production of the other. In the host, bacterial lipopolysaccharide (LPS) induces catecholamines and catecholamine-generating and degrading enzymes by alveolar macrophages and blood PMNs[222]. On the pathogen side, host microbiota is capable of producing biologically active, free catecholamines in the gut lumen[13b, 223], suggesting that host-microbiota-pathogen cross-talk occurs. NE affects enteric pathogen shedding in infected animals[131] and enhances *S.* Typhimurium-induced enteritis and bacterial replication in a bovine

ileal loop model[34]. Catecholamines have been shown to increase HGT in pathogenic and commensal bacteria[224]. *In vitro,* NE caused significant up-regulation in *E. coli* of *tra* genes G, I, J, R and Y, which are required for conjugative transfer of plasmid DNA[224]. NE-induced HGT could be reversed by adrenergic antagonists phentolamine and propranolol[224]. Phentolamine and propranolol have equal affinity to α1, α2 and β1, β2 receptors respectively. Propranolol is used to treat essential tremor in people and is used in animals to regulate heart rhythm. Adrenergic agonists such as ractopamine, zilpaterol, salbutamol, and terbutaline have structures similar to NE and Epi and are able to bind to host adrenergic receptors. Ractopamine and zilpaterol are used to increase muscle leanness in livestock by increasing lipolysis and myosin synthesis and decreasing lipogenesis[225]. Salbutamol and terbutaline are used to treat wheezing and shortness of breath associated with asthma, chronic obstructive pulmonary disease, bronchitis and emphysema. AAs may participate in bacterial signaling. For example, ractopamine use was associated with increased *E. coli* O157:H7 and *Salmonella* fecal shedding in sheep[226] and cattle respectively[227] indicating possible food safety implications. Possible participation of synthetic AAs in bacterial communication raises concerns regarding their use in animals and humans, although their effect on bacterial virulence and HGT needs to be further evaluated. The goal of this study was to determine the effect of adrenergic agonists on bacterial motility, growth and plasmid transfer.

5.3. Results

5.3.1. Effect of adrenergic agonists on bacterial growth and motility

Adrenergic agonists ractopamine, salbutamol and terbutaline did not significantly affect growth of *E. coli* and *S.* Typhimurium at concentrations ranging from 50 to 1000 µM (Figures 24 and 25).

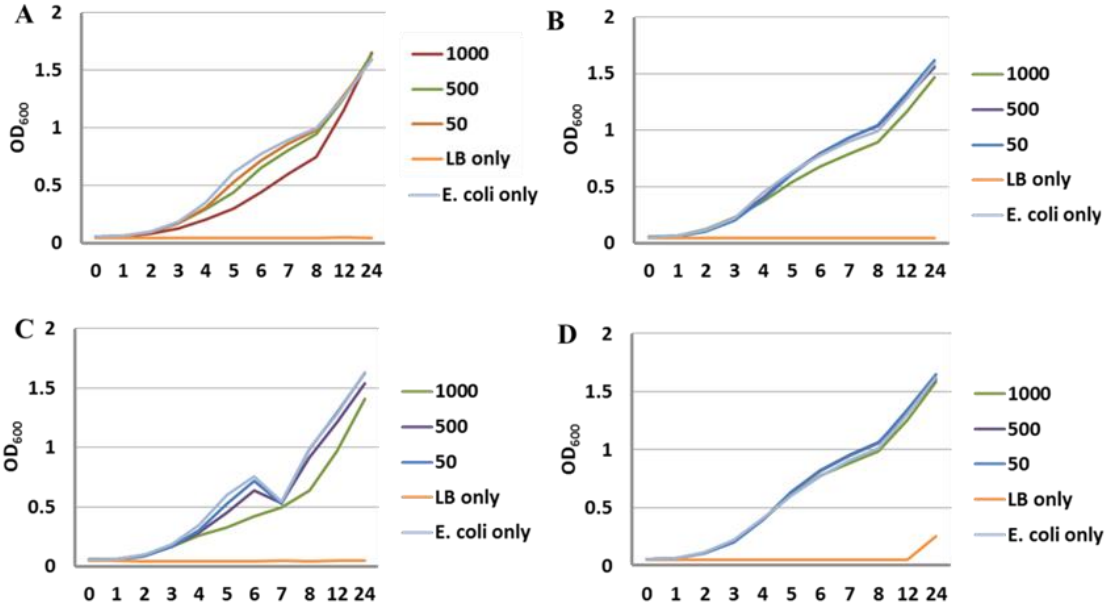

Figure 24: Growth of *E. coli* in presence of norepinephrine (A), salbutamol (B), ractopamine (C) and terbutaline (D).

As shown in previous studies, physiological concentrations of NE (50 µM) increased motility of *S.* Typhimurium in semi-solid medium (Fig. 26A). The metabolite of NE, DHMA, increases *S.* Typhimurium motility at 0.5 and 5 µM (Fig. 26B). Adrenergic agonists salbutamol and terbutaline did not have an effect on bacterial motility (Fig. 26 DE). Ractopamine decreased motility of *S.* Typhimurium at 50 and 1000 µM in semi-solid agar (Fig. 25C).

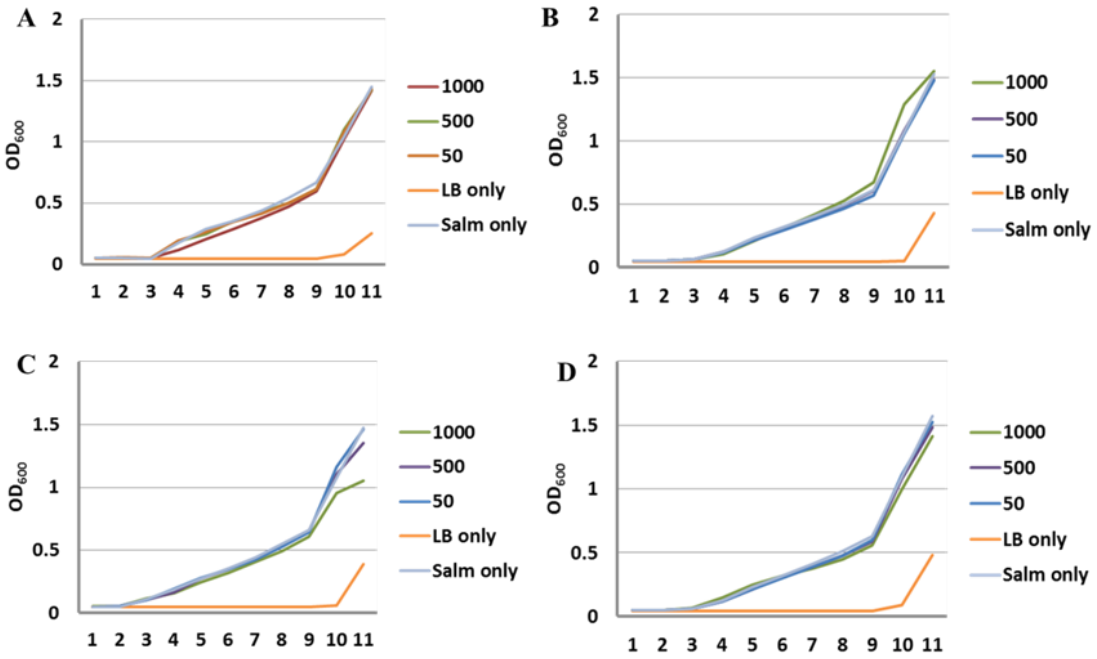

Figure 25: Growth of *S.* Typhimurium in presence of norepinephrine (A), salbutamol (B), ractopamine (C) and terbutaline (D).

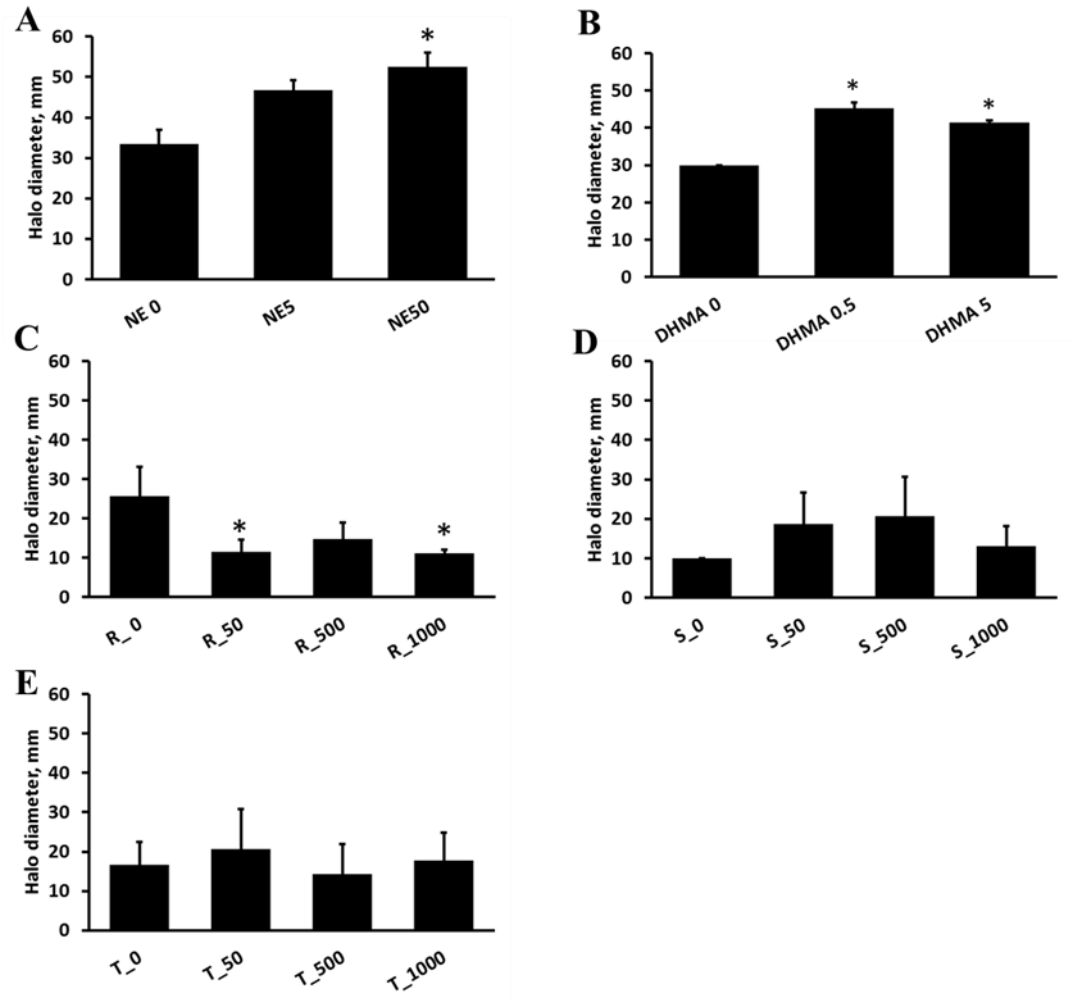

Figure 26: The effect of NE (A), DHMA (B), ractopamine (C), salbutamol (D) and terbutaline (E) on the motility of *S*. Typhimurium. Bars represent standard deviation. ****P* < 0.05 when compared to lack of treatment.

5.3.2. Effect of adrenergic agonists on horizontal gene transfer

We determined that 5 µM DHMA had a tendency to decrease HGT between *S.* Typhimurium and *E. coli* (P = 0.0776) at 4h post-mixing (Fig. 27). There was no effect of DHMA on HGT at 30 min, 1h, 2h or 6h post-mixing. We found a significant effect of the adrenergic agonist ractopamine on HGT at 50 µM and 150 µM. However, there was

no effect of ractopamine, salbutamol and terbutaline on HGT between *S.* Typhimurium and *E. coli* at predetermined time points (results not shown).

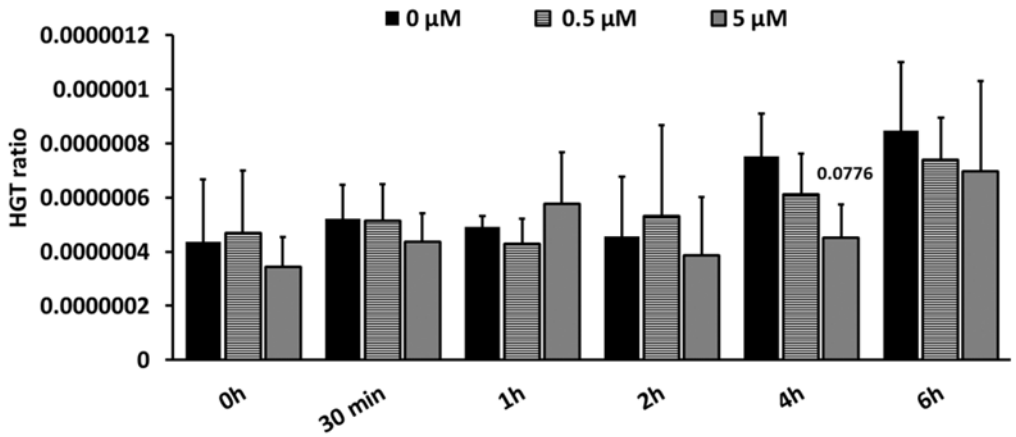

Figure 27: The effect of DHMA on plasmid transfer between donor *S.* Typhimurium and recipient *E. coli*. HGT ratio is calculated as ratio of number transconjugants to the number of recipients. Bars represent standard deviation.

5.4. Discussion

Antimicrobial resistance is an increasing problem worldwide in both humans and animals. Transmission of antimicrobial resistance genes from resistant bacteria to susceptible organisms occurs through mechanisms of HGT and is affected by multiple factors, including exposure to stress-hormones such as norepinephrine[224]. In mammals AAs have an affinity to the adrenergic receptors of the host and are widely used in animal production, human and veterinary medicine. It is known that synthetic adrenergic antagonists can inhibit NE-induced growth and HGT in bacteria[38, 224]. Synthetic AAs have structural similarity to catecholamines and are recognized by mammalian adrenergic receptors; therefore they could participate in bacterial signaling. We proposed

that AAs can participate in bacterial signaling, including stimulation of HGT and contributing to spread of antimicrobial resistance. The present study determined the lack of effect of the adrenergic agonists ractopamine, salbutamol and terbutaline on growth of pathogenic *S.* Typhimurium 5678 and commensal *E. coli* MG1655. It has been shown that growth-inducing properties of various additives depend greatly on the media used. For example, NE-induced growth is detected only in serum-enriched iron-deficient media, where NE makes iron available for bacterial use[228].

Next, we determined that the NE metabolite, DHMA increased bacterial motility in semi-solid media, which agrees with previous reports[40]. Additionally, DHMA had a borderline negative effect on HGT between *S.* Typhimurium and *E. coli* at 4h post-mixing. This suggests that Tsr-induced signaling may regulate virulence differently than NE-induced QseC/F signaling described in previous research[41].

Finally, the adrenergic agonists salbutamol and terbutaline had no effect on bacterial motility and plasmid transfer although ractopamine decreased *S.* Typhimurium swarming motility in semi-solid agar.

Different bacterial species and strains have various capacity for HGT[229]. It is possible that the donor-recipient pair we used was not the most efficient combination to study HGT, although this pair has been used previously (REF). Additionally, HGT is a relatively rare event, and the number of transconjugants is low (up to 100 CFU/mL). Therefore high variability of results may be due to difficulties in detection of transconjugants. It is possible, that a more efficient mating pair would result in increased

HGT efficiency and decreased variation. Additionally, *in vitro* experiments do not account for complex processes that occur in the gut.

Considering their hormone-like properties, adrenergic agonists could cause intestinal microbiota shifts which need to be evaluated. To date, the only study indicating the stimulatory effect of catecholamines on HGT was done *in vitro*[224]. It is difficult to predict the catecholamine concentration in the intestinal lumen in animals challenged intravenously. Additionally the half-lives of NE and Epi are short, making it difficult to evaluate their effects when given systemically. Synthetic adrenergic agonists have longer half-lives than NE and Epi and can be administered intragastrically, making their gut concentrations predictable. Additionally, these chemicals are extensively used in agriculture and medicine, and their effect on microbiota and antimicrobial resistance spread should be investigated.

5.5. Materials and methods

5.5.1. Bacterial growth conditions

Salmonella enterica serovar Typhimurium strain 5678[41] and *E. coli* MG1655 grown aerobically at 37°C with aeration in lysogeny broth (LB) containing 0.5% NaCl, 1% tryptone, 0.5% yeast extract), unless indicated otherwise. When necessary, antibiotics were used in the following concentrations: nalidixic acid 12 µg/mL, ampicillin 50 µg/mL (Sigma).

To assess growth rate overnight cultures of *S.* Typhimurium were diluted 1:100 and grown in LB broth with various concentrations of NE, DHMA, ractopamine,

salbutamol and terbutaline. Turbidity of the culture was measured every hour during over a 24 hour period at OD_{600}.

5.5.2. Assessment of bacterial motility

To determine the effect of NE, DHMA and AAs on motility early-log cultures of *S.* Typhimurium were grown on 0.3% LB agar impregnated with above additives at 37ºC. The diameter of bacterial halo was measured after 8h of incubation.

5.5.3. Bacterial mating

The rate of HGT between *Salmonella* and *E. coli in vitro* was determined using methods similar to previous study[224]. For liquid mating, overnight cultures of the donor *S.* Typhimurium 5678 and the acceptor *E. coli* MG1655 were inoculated into fresh pre-warmed LB at concentration 1:100 and incubated to reach OD_{600}=0.3. Donor and recipient were mixed at concentration 1:5. . Various concentrations of DHMA (0.5, 5 µM) and ractopamine (50, 150 µM) were added to the mixed cultures. Samples were be collected at 0h, 30 min, 1h, 2h, 4h, and 6h post-mixing, plated on LB agar plates with selective antimicrobials and screened for transfer of all the resistance markers encoded on the plasmid. Transconjugants were checked for the presence of the *blaCMY-2* gene by PCR. HGT ratios were determined by dividing CFUs/mL of transconjugant by CFUs/mL of recipient bacteria.

5.5.4. Statistical analysis

ANOVA followed by Tukey's T-test was performed (JMP, Version 11. SAS Institute Inc., Cary, NC, 1989-2007) to determine the difference in extracellular bacterial growth, motility and HGT.

6. AN ASSOCIATION BETWEEN TEMPERAMENT AND *E. COLI* O157:H7 SHEDDING IN BRAHMAN CALVES

6.1. Introduction

E. coli O157:H7 is a part of a normal flora in cattle, where the organism colonizes the colon and is shed in feces[230]. In people *E. coli* O157:H7 infection may lead to diarrhea, intestinal inflammation and 5-10% of patients develop hemolytic uremic syndrome[230]. The infectious dose for *E. coli* O157:H7 is only 100 colony-forming units (CFUs)[230]. The ingestion of the bacteria occurs through consuming meat, milk and produce which are contaminated with bovine feces[231]. Transmission to people can also occur via direct contact with an animal[231]. There are multiple factors that affect *E. coli* O157:H7 shedding. Several studies determined the association between a grain diet and increased *E. coli* O157:H7 shedding[232 233]. However some researchers have indicated that forage-fed cattle shed *E. coli* O157:H7 longer than grain-fed animals[234 235]. Transportation stress has been shown to increase *E. coli* O157:H7 shedding in finishing cattle[99a]. The stress hormones, norepinephrine (NE) and epinephrine (Epi) as well as dietary catecholamines, have been shown to increase growth of *E. coli* O157:H7 *in vitro* when grown in iron-limited media[236]. In the ligated ileal loop model NE increased *Salmonella enterica* replication and intestinal colonization[131]. In pigs handling stress was associated with increased shedding of total coliforms and *E. coli* O157:H7[237]. Therefore stress and the associated increase in gut catecholamine concentrations may lead to increased *E. coli* O157:H7 growth and shedding. The excitable temperament in cattle is

associated with increased serum cortisol and plasma norepinephrine concentrations[180b]. However the effect of temperament on the *E. coli* O157:H7 is not known. Therefore the objective of this study was to conduct a cross-sectional study to determine the association between temperament and *E. coli* O157:H7 prevalence in Brahman calves at weaning.

6.2.1. E. coli *O157:H7 isolation*

The *E. coli* O157:H7 isolation was performed as described previously[232]. Briefly, fecal samples were kneaded for 1 min, and 1 g of feces was added to 9 mL of gram-negative (GN) broth containing cefixime (0.5 mg/L), cefsulodin (10 mg/L), and vancomiycin (8 mg/L). The enrichment cultures were incubated overnight at 37 ^{0}C. Enriched samples were subjected to immunomagnetic bead separation followed by plating onto sorbitol-McConkey with cefixime (0.5 mg/L) and potassium tellurite (2.5 mg/L) (ct-SMAC). Up to 4 sorbitol-negative colonies were sub-cultured onto blood agar plates. Following overnight incubation colonies were selected for indole production and the latex-agglutination test with the O157 antigen. Positive isolates were inoculated into LB broth, grown overnight and stored at -80^{0}C.

6.2.2. Prevalence of E. coli *O157:H7 in Brahman calves*

E. coli O157:H7 was detected in 17 and 25 samples collected on d0 and d4, respectively. Twelve calves shed *E. coli* O157:H7 on both d0 and d4. There was a strong association of increased cortisol concentrations with temperament ($P < 0.05$) and sex (females 21.5±1.7 ng/mL, males 14.6±2.0 ng/mL, $P < 0.05$) (Table 12). These

findings support previous data regarding a positive association of serum cortisol concentration and temperament in *Bos indicus* calves[180b].

Table 12: Prevalence of *E. coli* O157:H7 at weaning and 4 days post-weaning.

	Calm	Intermediate	Temperamental	Total	Prob>ChiSq
Total	23	43	12	78	
Gender					
Male	15	13	5	33	0.2905
Female	8	30	7	45	0.5243
E. coli O157:H7 status					
Positive day 0	6	9	2	17	0.7975
Positive day 4	5	16	4	25	0.4366
Positive day 0 and day 4	3	7	2	12	0.7887

The serum cortisol concentration of the 12 calves shedding *E. coli* O157:H7 (16.9±2.6 ng/mL) did not differ ($P > 0.10$) from that of calves that were negative on either one or both of the sampling days. However, *E. coli* O157:H7 prevalence did not increase with the elevated serum cortisol associated with a more excitable temperament at weaning and post-weaning in Brahman calves. The prevalence of six major virulence genes (*fliC, stx1, stx2, eae, rbfE,* and *hlyA*) in *E. coli* O157:H7 isolates was determined by multiplex PCR[238] (Fig. 28). All of the 37 isolates carried the gene encoded for Shiga-toxin 2, while none of the isolates were positive for Shiga-toxin 1. Antimicrobial susceptibility of isolates was accessed by the automatic microdilution method. The majority of the isolates were susceptible to the antimicrobials included in the National Antimicrobial Resistance Monitoring System testing of Gram negative organisms with the exception of

the 55% of isolates that were resistant to sulfisoxazole. All of the isolates carried genes required for intimin and Shiga-toxin 2 production.

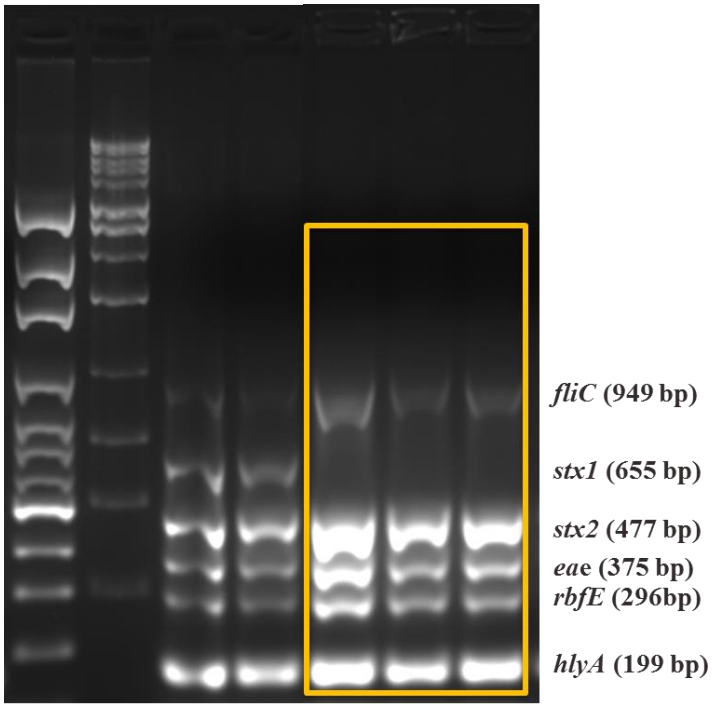

Figure 28: 42 isolates (100%) of *E. coli* O157:H7 positive for the intimin (*eae*) and Shiga-toxin 2 (*stx2* [5]) – producing genes.

Catecholamines have been shown to promote dose-dependent *E. coli* O157:H7 growth in media that resembles the intestinal environment[236a]. Transportation stress induced increased *E. coli* O157:H7 shedding in cattle of calm temperament[99a]. Based on these alternative hypotheses we speculated that temperament and associated differential catecholamine concentration in cattle affect *E. coli* O157:H7 growth and subsequent shedding. Alternatively, gut catecholamines could induce a shift in the microbiome composition to conditions favorable for *E. coli* O157:H7 persistence and growth. However, in the current study the proportions of calves shedding *E. coli* O157:H7 was

roughly equal among temperaments as determined by Chi-Square analysis. It is worth noting that *E. coli* O157:H7 prevalence is transient and can accumulate and dissipate within a 29 h period[239]. Hence cautious conclusions should be drawn if only prevalence data are available.

7. CONCLUSIONS AND FUTURE DIRECTIONS

Salmonella employs variety of factors to succeed in the mammalian GI tract. Among other tactics, *Salmonella* can intercept gut-microbiota communication and use signaling molecules to increase virulence and outcompete resident microbes. Additionally, *Salmonella* produces autoinducer 2 and autoinducer 3 which are involved in the regulation of virulence determinants such as invasion genes and flagella. In addition to QS molecules, virulence genes are regulates by other systems, including SirA/BarA-CsrA regulatory cascade, which is involved in carbon metabolism and SPI-1 regulation in *Salmonella*[63b]. In present study we determined that SirA/BarA-CsrA regulatory cascade regulates AI-2 QS in *Salmonella*. It has been known that two-component SirA/BarA system, which senses SCFAs, regulates CsrA activity through small regulatory RNAs *csrB* and *csrC*[150a]. In this work we determined that global regulator CsrA directly binds to *lsr* promoter thus repressing it, indicating an interaction between carbon regulation and AI-2 QS in *S.* Typhimurium. Additionally, we identified an interaction between AI-2 and AI-3 QS systems. We determined that AI-3 response regulator QseF suppresses *lsr* expression and AI-2 internalization. Catecholamine horrmone NE was also found to suppress *lsr* operon expression and increase AI-2 internalization in *S.* Typhimuruim. Thus we discovered that AI-3/Ne/Epi sensing system regulates *lsr*-dependent AI-2 QS in *Salmonella*.

Bacteria use QS to survive in the gut, and pathogenic *S.* Typhimurium uses QS to outcompete resident microbiota and increase virulence. In addition to self-produced

molecules, *S.* Typhimurium can sense host catecholamines Epi and NE, which signal through AI-3/NE/Epi signaling system and increase growth, motility, biofilm formation and plasmid transfer[38, 41, 126, 240]. Synthetic adrenergic agonists have structure similar to catecholamines, and have been speculated to promote bacterial survival and shedding in feces[226-227]. However, we determined that adrenergic AAs salbutamol and terbutaline do not increase growth, motility and plasmid transfer. Ractopamine did not affect growth and plasmid transfer, but decreased motility of *S.* Typhimurium.

Weaning is a stressful event and is associated with increased concentrations of stress hormone cortisol and catecholamines NE and Epi[241]. In ruminants weaning is characterized by transitioning from monogastric type of digestion to ruminal fermentation and has been associated with significant changes in ruminal and fecal microbiota[188 189]. We determined that weaning had a tremendous effect on fecal microbiota as well as fecal and serum metabolites in Brahman calves, where about 1/3 of detected fecal and serum compounds was differentially abundant between weaning and 4 days post-weaning. We have also found that sex was associated with differentially abundant microbiota and fecal metabolites after weaning, indicating a possible stress-exacerbated differences between pre-pubertal males and females. Excitable temperament is associated with increased resting serum cortisol concentrations, and has been associated with differential immune response, glucose tolerance and pathogen shedding[99a, 100, 206]. We had an unique opportunity to study an effect of the temperament on the microbiota and metabolites in purebred Brahman calves. We determined that temperament was associated with differentially abundant microbiota at weaning and

metabolites at- and after weaning. Therefore weaning- or temperament- dependent increased concentrations of stress hormones are associated with changes in microbial and metabolic composition in calves. Additionally, we found that temperament was not associated with *E. coli* O157:H7 shedding at weaning or 4 days after weaning.

In this study we investigated regulation of *Salmonella* AI-2 and AI-3 quorum sensing as well as effect of catecholamine NE on bacterial communication. We identified an interaction between AI-2 QS and carbon metabolism as well as a cross-talk between AI-2 and AI-3 QS systems. We determined that, unlike NE[38, 41], synthetic adrenergic agonists do not enhance *S.* Typhimurium growth, motility and plasmid transfer *in vitro*, suggesting that synthetic adrenergic agonists do not increase virulence of Entrobacteiaceae. Finally, we determined that weaning stress and excitable temperament are associated with shifts in microbial and metabolic composition, indicating stress hormone-dependent changes in gut-brain interaction.

Thus, in this study, we have elucidated some mechanisms of intraspecies and interkingdom communication, which are common in a metabolite-rich gut environment, and which allow for multidirectional communication between pathogen, microbiota and host.

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