An Analysis of *Salmonella* from Molecular, Cellular, and Food Safety Perspectives.

by

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Abstract

Salmonella is a major foodborne pathogen in both developing and developed countries and is responsible for a range of diseases including enteric fever, gastroenteritis, and bacteremia. Despite advancements in food safety strategies, *Salmonella* illness continues to substantially contribute to foodborne related hospitalization and deaths in both Canada and the United States. While a majority of these illnesses are associated with the mishandling of raw meat, a large portion are related to outbreaks from incidental contamination of food products. In Canada, these contaminated products are recalled from retailers by the Canadian Food Inspection Agency to prevent the transmission of *Salmonella*. However, not all *Salmonella* contribute to severe health outcomes as there is a large degree of genetic heterogeneity among the 2600 serovars within the genus. This range in genetic variability across *Salmonella* serovars is linked to numerous genetic elements that dictate virulence. How these elements collectively constitute *Salmonella* disease is not completely understood. Several genetic elements encode highly studied virulence factors, such as *Salmonella* pathogenicity island 1 and 2, with well documented contributions to pathogenesis. However, many genetic elements implicated in *Salmonella* virulence remain uncharacterized. Identifying how these potential virulence factors contribute to *Salmonella* disease is essential to understanding the virulence disparity between serovars. In this thesis I analyze *Salmonella* from molecular, cellular, and food safety perspectives to investigate some of these research questions. First, I examine the contributions of *Salmonella* to microbial-related recalls in Canada from 2000-2017 in which I highlight recent increases in *Salmonella*-related recalls involving fruits, vegetables, and leafy greens. Second, I discuss my research on evaluating protozoa as a screening model for *Salmonella* virulence and present the *Acanthamoeba* screening model I developed to characterize the virulence of clinical and environment isolates of *Salmonella*. Third, I explore the role of the E3 ubiquitin ligase effector SspH1 during *Salmonella* infection and demonstrate that it stimulates degradation of the host kinase PKN1. In addition, I investigate the prevalence of *sspH1* in a large collection of *Salmonella* isolates (the Syst-OMICS consortium) and reveal a potentially new SspH1-related protein. Lastly, I propose future directions related to the work presented in my thesis.

List of Abbreviations Used

ADP	Adenosine diphosphate
AF1/2/3	Apoptotic fragment
Akt	Protein kinase B
AP-1	Activator protein 1
AP-MS	Affinity-purification mass spectroscopy
AR	Androgen receptor
ARG	Androgen regulated genes
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AvrA	Avirulence protein A
AvrPtoB	Avirulence protein B from *Pseudomonas syringae pv tomato*
bap	Biofilm associated protein
BEL	Bacterial encoded E3 ubiquitin ligase
BioID	Biotin identification
BLOC-2	Biogenesis of lysosome-related organelles complex 2
BSA	Bovine serum albumin
C4b	Complement component 4b
C9	Complement component 9
Caspase	Cysteine-dependent aspartate-directed proteases
CFIA	Canadian Food Inspection Agency
CFU	Culture forming units
CRL	Cullin-RING ligase
C-terminus	Carboxyl-terminus
Dictyostelium	*Dictyostelium discoideum*
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DT2	Definitive type 2
DTT	Dithiothreitol
DUB	Deubiquitinase
EGFR	Epidermal growth factor receptor
EHEC	Enterohaemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ERdj3	Endoplasmic reticulum DnaJ homologue 3
ERK	Extracellular signal-regulated kinase
F-actin	Filamentous actin
FBS	Fetal bovine serum
FBX022	F-box only 22
FDA	Food and Drug Administration
FRT	Flippase recognition target

G-actin	Globular actin
GAP	Guanosine triphosphatase activating protein
GBP	Guanylate-binding protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GI	Gastrointestinal
GipA	Growth in Peyer's Patches A
gogA/B	*Gifsy-1* gene A/B
GPA	Gentamicin protection assay
gtgA/E	*Gifsy-2* gene A/E
GTP	Guanosine triphosphate
H antigen	Flagellar antigen
HECT	Homologous to the E6-AP Carboxyl Terminus
hilA/D/C	Hyperinvasion locus
HlyE	Hemolysin E
HOIL	Heme-oxidized IRP2 ubiquitin ligase
HOIP	HOIL interacting protein
HOPS complex	Homotypic fusion protein sorting
HPS3	Hermansky–Pudlak syndrome 3 protein
HR1a/b/c	Homology region 1
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IBR	In-between RING
IFN-β	Interferon-β
IKK	IκB kinase
IL	Interleukin
IMS	Issue Management System
Inv	Invasion protein
ip	Intraperitoneal
IP	Immunoprecipitation
IpaH	Invasion plasmid antigen H
IQGAP1	IQ motif containing GTPase activating protein 1
iv	Intravenous
Iκβα	NF-κB inhibitor-alpha
JNK	c-Jun N-terminal kinase
K0	Light lysine
K8	Heavy lysine
KD	Knockdown
kDa	Kilodalton
LAMP1	Lysosomal-associated membrane protein 1
LB	Lysogeny broth
LDH	Lactate dehydrogenase
LoiA	Low oxygen induced factor A

LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LUBAC	Linear ubiquitin chain assembly complex
M cell	Microfold cell
MAPK	Mitogen activated protein kinase
MDS	Multidimensional analysis
MgtB/C	Magnesium transport protein
MLK	Mixed lineage kinase
MLST	Multi-locus sequence typing
MLTK	MLK-like mitogen activated protein triple kinase
MLVA	Multiple-locus variable number of tandem repeats analysis
mMHCII	Mature major histocompatibility complex II
MOI	Multiplicity of infection
MPR	Mannose-6 phosphate receptor
mPYG	Modified peptone-yeast extract-glucose
MR	Mineralocorticoid receptor
NEDD4/8	Neural precursor cell expressed developmentally down-regulated protein
NEL	Novel E3 ubiquitin ligase
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NleE/L/G	Non-locus of enterocyte effacement encoded effector
NLR	Nod-like receptor
NLRP3	Nod-like receptor family pyrin domain containing 3
NOD1	Nucleotide-binding oligomerization domain-containing protein 1
NPR1	Non-expresser of PR gene 1
Nramp1	Natural resistance-associated macrophage protein 1
Nt	Non-targeting
NT	Non-typhoidal
N-terminus	Amino-terminus
ORF	Open-reading frame
OSBP1	Oxysterol binding protein 1
OspI/Z	Outer *Shigella* protein I
p-Akt	Phospho-Akt
PAMP	Pathogen associated molecular pattern
PAS	Page's amoeba saline
PBS	Phosphate-buffered saline
PCB	Plate count broth
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase 1
pef	Plasmid encoded fimbriae
PEI	Polyethylenimine
PFGE	Pulse-field gel electrophoresis
PHAC	Public Health Agency of Canada

PI	Propidium iodide
PI(3, 4, 5)P$_3$	Phosphatidylinositol (3, 4, 5)-triphosphate
PI(4)P	Phosphatidylinositol (4)-phosphate
PIP	Phosphatidylinositol phosphate
PipA/B/B2	Pathogenicity island encoded protein
PKN1/2	Serine/threonine-protein kinase N1/2
PLEKHM1/2	Pleckstrin homology domain-containing family M member 1/2
PMA	Phorbol 12-myristate-13-acetate
PR	Progesterone receptor
prpZ	Protein phosphatase Z
PRR	Pattern recognition receptor
PTM	Post-translational modification
PUP	Prokaryotic ubiquitin-like protein
PVDF	Polyvinylidene fluoride
Rab-GTPase	Rab guanosine triphosphatase
RBR	RING-in-between-RING
RCC	Regulators of chromatin condensation 1
rck	Resistance to complement killing
Rho-GTPase	Rho-Guanosine triphosphatase
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation assay
RLD	RCC-like domains
RPM	Revolutions per minute
rsk	Reduced serum killing
RSK1/2	Ribosomal S6 kinase ½
RTE	Ready-to-eat
RTK	Receptor tyrosine kinase
saf	*Salmonella* atypical fimbriae
SalFos	*Salmonella* Foodborne Syst-OMICS
SAMP	Small archaeal modifier protein
SarA	*Salmonella* anti-inflammatory response activator
SCF	SKIP1-Cull1-F-box protein
SCV	*Salmonella* containing vacuole
SCX	Strong cation exchange
SdiA	Suppression of cell division inhibition A
SDS	Sodium dodecyl sulphate
sef	*Salmonella* Enteritidis fimbriae
shRNA	short hairpin RNA
SIF	*Salmonella* induced filament
SifA	*Salmonella* induced filament protein A
SiiE	*Salmonella* intestinal infection protein E
SILAC	Stable isotope labelling by amino acids in cell culture
Sip	*Salmonella* invasion protein

SKIP1	S-phase kinase-associated protein 1
SlrP	*Salmonella* leucine-rich repeat protein
SM	Standard medium
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptor
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
SNX	Sorting nexin
SOD	Superoxide dismutase
Sop	*Salmonella* outer protein
SPI	*Salmonella* pathogenicity island
SptP	*Salmonella* protein tyrosine phosphatase
spv	*Salmonella* plasmid virulence
srgA	SdiA regulated gene A
Sse	*Salmonella* secretion effector
SspH1/2	*Salmonella* secreted protein H1/2
SsrAB	*Salmonella* secretion regulator AB
ST313	Sequence type 313
Ste	*Salmonella* translocated effector
T1SS	Type one secretion system
T3SS	Type three secretion system
T3SS-1	SPI-1 type three secretion system
T3SS-2	SPI-2 type three secretion system
T5SS	Type five secretion system
T6SS	Type six secretion system
TAB2/3	TGF-β-activated kinase 1 MAPK kinase 7-binding protein 2/3
TaiA	Typhi-associated invasin A
TBK1	TRAF family member-associated NF-κB activator-binding kinase 1
TBST	Tris-buffered saline with Tween-20
tcf	Typhi colonizing factor
TGF	Tumor growth factor
TH1	T helper 1
TLR4	Toll-like receptor 4
TMEM127	Transmembrane protein 127
Tn	Transposon
Tn-seq	Tn sequencing
TNF	Tumor necrosis factor
TRAF6	Tumor necrosis factor receptor associated factor 6
TRIM	tripartite motif
Trx	Thioredoxin
Ub	Ubiquitin
UBA1/6	Ubiquitin-like modifier-activating enzyme
Ubact	Ubiquitin bacterial
UBAIT	Ubiquitin activated interaction trap

Ubc	Ubiquitin conjugating enzyme
U-box	UFD2 homology domain
UPS	Ubiquitin proteasome system
Vi antigen	Capsular antigen
viaB	Vi antigen B
wgMLST	whole genome MLST
WGS	Whole genome sequencing
WHO	World Health Organization
WHOCC-Salm	WHO Collaborating Centre for Reference and Research on *Salmonella*
WWP2	WW domain-containing protein 2
YlrA/C	*Yersinia* leucine-rich repeat protein
YopM	*Yersinia* outer membrane protein M

Acknowledgments

Chapter 1: Introduction

1.1. The Genus *Salmonella*

The genus *Salmonella* represents a collection of genetically related Gram-negative intracellular bacteria that are organized into serotypes (more commonly referred to as serovars) at the lowest level of classifications. There are over 2600 *Salmonella* serovars currently identified and among them exists a range of genetic diversity (1). Despite their differences, all *Salmonella* serovars are transmitted via the fecal-oral route, commonly through the ingestion of contaminated food or water. Upon ingestion, *Salmonella* enter the gastrointestinal (GI) tract of the host where they initiate infection. The severity of infection varies between serovars, ranging from a self-limiting infection in the GI tract (gastroenteritis) to bacteremia and systemic illness. *Salmonella* serovars also differ greatly in their host range causing infection in humans and a variety of animals. To help make sense of the great diversity among the many *Salmonella* serovars, the World Health Organization Collaborating Centre for Reference and Research on *Salmonella* (WHOCC-Salm) has developed a multi-level nomenclature system to classify serovars into related groupings (1).

1.1.1. *Salmonella* Nomenclature

The genus *Salmonella* is named after Daniel E. Salmon, an American bacteriologist, who first isolated the bacteria, *Salmonella choleraesuis*, from a porcine intestine in 1885 (2). Since its discovery, the WHOCC-Salm have identified thousands of new variations of *Salmonella* and have classified the isolates using serotyping analysis. *Salmonella* serotyping examines antibody-epitope interactions on bacteria indicating the presence of specific surface antigens. Fritz Kauffman, the first lead scientist of the

WHOCC-Salm, introduced the first iteration of a *Salmonella* classification system, involving early work by Bruce White, based on serotyping analysis in 1931 (2). The initial Kauffman-White scheme classified each antigenically distinguishable type of *Salmonella* by its expression of specific O (O polysaccharide of lipopolysaccharide, LPS), H (flagellar – monophasic and diphasic), and Vi (capsular) antigen. The scheme also defined each unique antigenic formula as a separate *Salmonella* species, designated by a name usually indicative of the associated diseases, their geographic origins, or their common habitats (3). Kauffman later supplemented the scheme with biochemical tests to divide the *Salmonella* genus into subgenera to improve the taxonomical classification of serotypes (4); although, these subgenera were rearranged through studying the genetic relatedness of isolates.

In 1973, Falkow and colleagues determined through DNA-DNA hybridization that nearly all identified *Salmonella* serotypes and subgenera were related at a species level (5). Since *S. choleraesuis* was the first species identified, it became the singular species name for nearly all serotypes (6). This change in nomenclature lead to confusion considering *S. choleraesuis* possessed different biochemical characteristics than some of the serotypes classified under it (7); additionally, "Choleraesuis" was also used as a serotype name (8). As a result, Leon Le Minor and Michal Y. Popoff, the second and third lead scientists for the WHOCC-Salm, proposed *Salmonella enterica* as a species name replacement as it was not a designated serotype name. Le Minor and Popoff further classified the *Salmonella enterica* species into seven subspecies, distinguishing *Salmonella* serotypes based on genetic subgrouping and biochemical characteristics (8,9). These seven subspecies (*enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb),

houtenae (IV), *bongori* (V), and *indica* (VI)) formed the basis of current *Salmonella* nomenclature under the *enterica* species, with the single exception of subspecies V. DNA hybridization experiments performed by Reeves *et al.* determined that the *bongori* subspecies evolved separately from all other *Salmonella* subspecies, but shares enough taxonomic characteristics to be classified under the genus *Salmonella* (10). As a result, the WHOCC-Salm elevated the *Salmonella* subspecies V to its own species, *Salmonella bongori* (11).

The WHOCC-Salm currently uses the White-Kauffman-Le Minor scheme, the most recent *Salmonella* antigenic classification system, to characterize newly isolated *Salmonella* serotypes into their corresponding subspecies (Figure 1.1). The updated scheme outlines the accepted nomenclature for *Salmonella* serotypes where genus, species, subspecies, and serotype (eg. *S. enterica* subsp. *enterica* ser. Typhimurium) or genus and serotype (eg. *Salmonella* ser. Typhimurium), at a minimum, must be stated (1). The scheme also uses serovar in place of serotype in accordance with the International Code of Nomenclature of Bacteria (12). The White-Kauffman-Le Minor scheme is supplemented and revised every few years to include newly classified serovars and to update serotyping criteria. New serovars classified under the subspecies *enterica* are designated by a name usually related to the geographical location where the serovar was isolated, while those classified under the other subspecies and *S. bongori* are referred to by their corresponding antigenic formula (1).

Within serovars there has been further characterization of variation using phage type analysis and multi-locus sequencing type (MLST). Phage type analysis identifies a
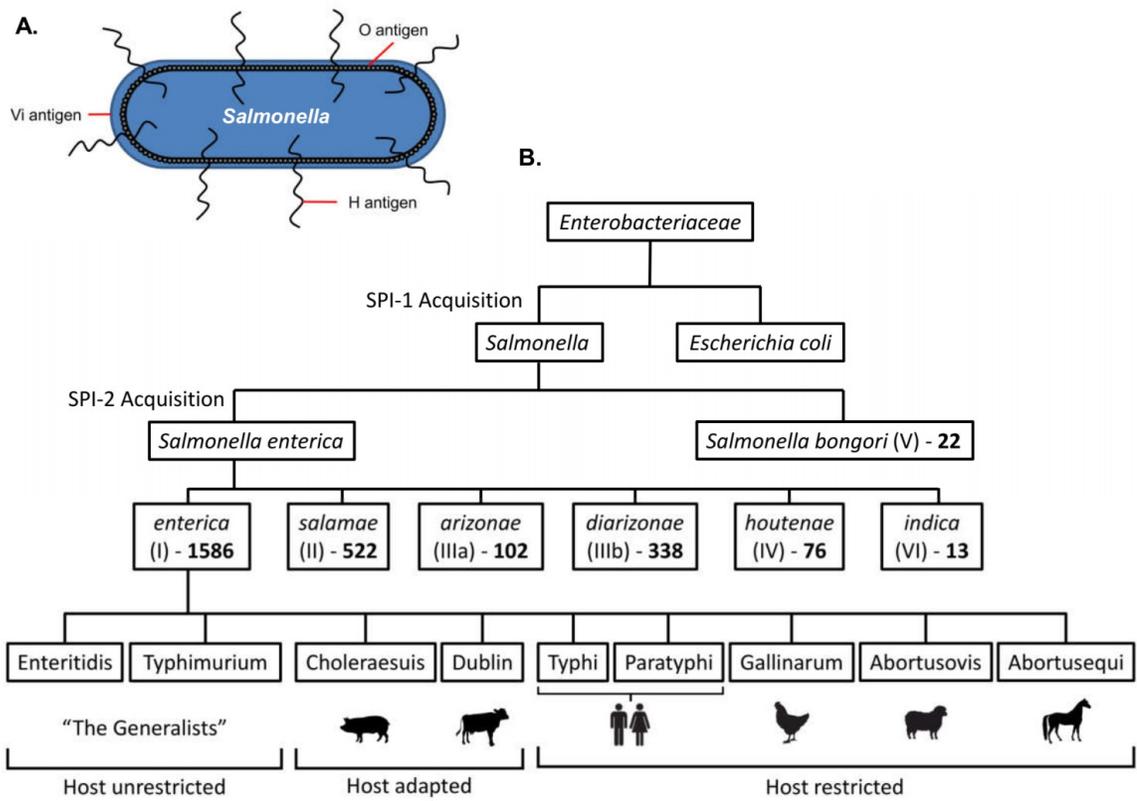
Figure 1.1: White-Kauffman-Le Minor *Salmonella* classification scheme.
(A) *Salmonella* are classified into serovars based on variations in LPS (O), flagellar (H), and capsular (Vi) antigens. Biochemical tests and phage typing are also performed to supplement antigenic subtyping of isolates. (B) The genus *Salmonella* diverged from a common *Enterobacteriaceae* ancestor shared with *Escherichia coli* through acquisition of *Salmonella* pathogenicity island-1 (SPI-1). SPI-1 granted *Salmonella* the ability to internalize into cells and establish gastrointestinal infections. The genus later separated into two species with *Salmonella enterica* acquiring SPI-2, enabling systemic pathogenesis within hosts, while *Salmonella bongori* (formerly known as subspecies V) did not. *S. enterica* diverged further into separate subspecies with *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) all mainly associated with endothermic vertebrates like *S. bongori*, whereas subspecies *enterica* (I) expanded into ectothermic hosts. Within the various species and subspecies, *Salmonella* are classified into serovars based on serotyping analysis outlined by the White-Kauffman-Le Minor classification system. Furthermore, *S. enterica* subsp. *enterica* serovars are grouped according to the host range and elicited disease, as some serovars are unrestricted in host specificity, some are more adapted to certain hosts than others, and some are completely restricted to specific hosts. The current total number of serovars in each subspecies (or species for *S. bongori*) as per the most recent White–Kauffmann–Le Minor scheme supplement (13) are written in bold. Figure modified from Gal-Mor, 2019 (14).

profile of phages that can infect *Salmonella*. In *Salmonella* ser. Typhimurium, phage typing has identified isolates with distinct genetic differences, referred to as pathotypes, coinciding with changes in disease outcomes (15). Additionally, multiple groups have identified new phylogenetic groups of *S. enterica* separate from the known subspecies through MLST and whole genome sequencing (16,17). The WHOCC-Salm classification system currently does not include the newly identified phylogenetic groups of *S. enterica* or Typhimurium pathotypes. While serotyping continues to play a critical role in *Salmonella* classification, future Kauffmann-White-Le Minor scheme revisions will reflect insights gained from new serovar characterization techniques (13).

1.1.2. *Salmonella* Host Range

There is great diversity between species, subspecies, and serovars related to host range across the genus *Salmonella*. From a broad view, *Salmonella* host range is often divided into two groups, endotherms and ectotherms (18). These two groups typically align with the antigenic classification of serovars as *S. enterica* subsp. *enterica* serovars primarily infect endotherms, while serovars from *S. bongori* and the non-*enterica* subspecies are typically found in ectotherms. However, this premise is not absolute, as both serovar groups can infect either temperature type (18). Where the serovar groups truly differ is in their capacity to cause systemic disease. In endotherms, *S. bongori* and non-*enterica* subspecies commonly produce an infection limited to the GI tract, whereas *S. enterica* subsp. *enterica* serovars infection can potentially spread throughout the host (19). This divergence of disease severity is reflected in the evolutionary events that divide the *Salmonella* species and subspecies (Figure 1.1B).

The speciation of *S. enterica* from *S. bongori* is attributed to the acquisition of *Salmonella* pathogenicity island 2 (SPI-2), which carries genes important for intracellular survival (20). However, the acquisition of SPI-2 alone does permit systemic infection in endotherms. *S. enterica* subsp. *arizonae* was the first subspecies to diverge from *S. bongori*, followed by *houtenae, diarizonae*, *salamae,* and *indica*; all of which possess SPI-2 and are associated with ectotherm infection (21). *S. enterica* subsp. *enterica* diverged last in the *enterica* subspecies and represents the lone subspecies associated with systemic infection in endothermic hosts. This divergence of *S. enterica* subsp. *enterica* into endotherms coincided with the acquisitions of genes important to invasion and survival in deep tissues, metabolism, and intercellular communication (15). Numerous studies have also demonstrated that non-*enterica* subspecies are incapable of survival or replicating within mammalian macrophages (22,23). The transition to endotherms for *S. enterica* subsp. *enterica* serovars likely required adaptation to a more complex immune system as gut-associated immunity in ectotherms is less complex (24). Nonetheless, many of the genetic difference between *S. enterica* subsp. *enterica* and the non-*enterica* subspecies are found within hypothetical genes or genes with putative functions (15). As a result, our understanding of the transition of *Salmonella* into endothermic hosts remains incomplete.

The diversity of *Salmonella* host range is also found between serovars of *S. enterica* subsp. *enterica*. This diversity in serovar-associated host range is separated into three categories: restricted, adapted, and unrestricted (19). Restricted host range refers to *Salmonella* that exclusively cause disease in a single host species. Both *Salmonella* ser. Typhi and Gallinarum are serovars with restricted host range as they only cause severe

systemic disease in humans and poultry, respectively (25). Adapted host range refers to *Salmonella* that cause disease in multiple species, but are most prevalent in a single host species. For example, *Salmonella* ser. Choleraesuis and Dublin primarily cause systemic disease in pigs and cattle, respectively, but can also infect humans and other mammals and are considered the two most notable *Salmonella* serovars with adapted host range (26,27). *Salmonella* serovars that infect numerous host species are categorized with unrestricted host range. The two most common *Salmonella* serovars with unrestricted host range are *Salmonella* ser. Typhimurium and Enteritidis, which can infect and cause disease in humans, cattle, pigs, and chickens (28); although, infection is largely self-limiting (29). Diversity in host range is also observed within serovars. Although the serovar Typhimurium infects numerous host species, the definitive type 2 (DT2), a pathotype, is host restricted to feral pigeons, despite its classification in a unrestricted host serovar (30). Relating serovar classification to host range is still useful as *Salmonella* surface antigens are major targets of host immune responses(31) and the Vi antigen is primarily found in the human restricted serovar Typhi (32). However, there are many other factors that contribute to *Salmonella* host specificity. With the advancement of genome analysis, correlating serovars with host range may lose value as we identify the genetic factors responsible for *Salmonella* host adaptation.

The factors that contribute to *Salmonella* adaptation to a specific host species also appear to influence *Salmonella* disease severity. Both *Salmonella* ser. Typhimurium and Enteritidis exhibit low specificity for host species (28), while their infections are largely restricted to the GI tract (33). Conversely, *Salmonella* ser. Typhi and Gallinarum both produce severe systemic disease in their respective hosts, but do not establish infection in

other species (25). The factors contributing to these differences in serovar host range are similar to those associated with the transition of *Salmonella* into endothermic hosts. Host restricted serovars, such as Typhi and Gallinarum, possess strict nutritional requirements relative to serovars with unrestricted host range, suggesting a relationship between the development of auxotrophy and host adaptation (19). Adaptation to a single host species also coincides with developing resistance to species-specific immune systems. For example, host restricted serovars, such as *Salmonella* ser. Typhi and Gallinarum survive better in macrophages and invade and replicate in deeper tissues in their respective hosts relative to other species (34,35). Alternatively, serovars with unrestricted host range are associated with severe systemic disease in early-age animals with immature immunity, but are restricted to the GI tract in adult animals with fully mature immune systems (29). A genome wide comparison of serovars associated with either GI infection or systemic disease in humans also demonstrated that downregulation of macrophage apoptosis, important for replication and dissemination of *Salmonella*, is highly represented in the latter (36). Altogether, adaptations in metabolism and survival in macrophages appear to correlate with the development of restricted host range.

1.1.3. *Salmonella* Infection Clinical Manifestations and Disease Progression

Infection with *S. enterica* in humans cause four distinct clinical manifestations: gastroenteritis, bacteremia, enteric fever, and asymptomatic carrier state. Infection with either a host unrestricted serovar, such as Typhimurium or Enteritidis, or a host adapted serovar, such as Dublin and Choleraesuis, commonly produce gastroenteritis in humans, which is characterized by nausea, vomiting, diarrhea, abdominal cramps, and fever (37). These serovars are referred to as non-typhoidal (NT) *Salmonella* as they usually remain

in the human GI tract. *Salmonella*-related gastroenteritis also has the potential to progress to bacteremia depending on the health of the host and on the pathogenicity of the serovar (33). Alternatively, infection with a human restricted serovar, such as Typhi or Paratyphi (also referred to as typhoidal *Salmonella*), causes enteric fever in humans, characterized by a sustained fever coupled with chills, abdominal pain, rash, heptosplenomegaly, nausea, headaches, and anorexia (37). Unlike NT-gastroenteritis, enteric fever is associated with the dissemination of *Salmonella* throughout the host. Systemic spread of typhoidal *Salmonella* will occasionally produce a chronic carrier state in humans, where *Salmonella* is asymptomatically shed in host feces for up to a year or longer following resolution of the initial infection (38). A chronic carrier state related to NT *Salmonella* is typically not observed as *Salmonella* is no longer detected in host stool after twelve days (39). While NT and typhoidal *Salmonella* present different clinical manifestations, both groups initiate infection in the GI tract.

Salmonella infection begins through the consumption of contaminated food or water. For gastroenteritis, an infectious dose of 10^3 NT *Salmonella* is enough to cause disease within a 48-hour period (40), while a dose of 10^5 typhoidal *Salmonella* will induce enteric fever within two weeks (41). Ingested *Salmonella* migrate to the intestinal mucosa where they interact with the intestinal epithelium and exit the lumen via three distinct pathways: microfold (M) cell transcytosis into the lamina propria, dendritic cell phagocytosis, and induced phagocytosis at apical membrane of enterocytes. Upon entering the lamina propria, *Salmonella* are phagocytosed by mucosal macrophages and additional dendritic cells (42). These interactions that take place at the intestinal mucosa

between *Salmonella* and the host are where NT-gastroenteritis and enteric fever diverge (Figure 1.2).

At the onset of NT-gastroenteritis, toll-like receptors (TLRs) on enterocytes and mucosal macrophages recognize pathogen-associated molecular patterns (PAMPs) from *Salmonella*, such as flagellin and LPS, stimulating a pro-inflammatory response and the production of the neutrophil chemotactic factor interleukin 8 (IL-8) (43). Internalized NT *Salmonella* establish a replicative niche in enterocytes and trigger apoptosis in mucosal macrophages, further promoting inflammation (44). Subsequent neutrophil recruitment and inflammation, both hallmarks of NT-gastroenteritis, induce fluid accumulation and tissue injury at the site of infection. This disruption of the intestinal epithelium releases fluid from the lamina propria into the intestinal lumen, which clinically presents as diarrhea (45). Activation of T helper 1 (T$_H$1) immunity later combines with neutrophils and activated macrophages to contain and ultimately clear the NT *Salmonella* infection (46). However, failure to restrict the infection to the GI tract can lead to bacteremia via the spread of NT *Salmonella* from the mesenteric lymph nodes into the host circulatory system (47). Dissemination of NT *Salmonella* through the blood stream is linked to numerous severe focal infections including, but not limited to, meningitis, mycotic aneurysm, and osteomyelitis (48). Whereas NT-gastroenteritis is usually self-limiting, NT-bacteremia is potentially lethal and is treated with antibiotics. Additional complications occasionally arise following bacterial clearance in the form of post-infection sequela. These complications manifest as reactive arthritis, inflammatory bowel disease (IBD) or neuronal damage associated with Guillain-Barré and Miller-Fisher
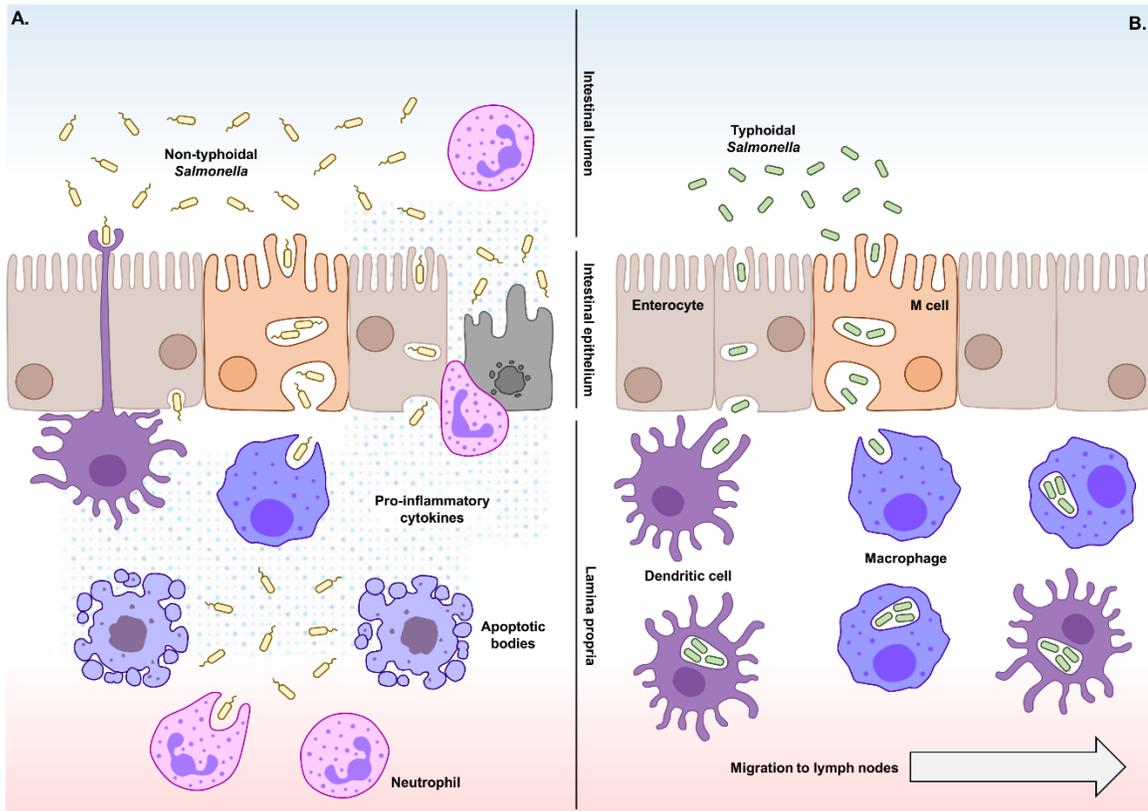
Figure 1.2: Gastrointestinal invasion during typhoidal and non-typhoidal _Salmonella_ infection.

Salmonella infection requires invasion of host cells in the gastrointestinal tract following ingestion. From the intestinal lumen, _Salmonella_ cross the intestinal epithelium boarder into the lamina propria via three mechanisms: Microfold (M) cell mediated transcytosis, induced phagocytosis in epithelial cells, and dendritic cell antigen sampling. Once inside the lamina propria, the type of _Salmonella_ dictates the corresponding immune response. (A) Non-typhoidal (NT) _Salmonella_ invasion is characterized by the secretion of pro-inflammatory cytokines, specifically IL-8, and recruitment of neutrophils. Pro-inflammatory cytokines are secreted in response to pattern recognition receptor (PRR) binding to pathogen associated molecular patterns (PAMPs), such as flagellin and LPS. Phagocytosed-NT _Salmonella_ also stimulate inflammation following induction of apoptosis in macrophages, dendritic cells, and intestinal epithelial cells. Infection of epithelial cells compromises the integrity of the intestinal boarder, which is exacerbated further by the influx of fluid accompanying inflammation. (B) In contrast to NT _Salmonella_, typhoidal _Salmonella_ elicit a weaker immune response due to a lower flagellin expression and a capsule that masks PAMP detection. In the lamina propria, typhoidal _Salmonella_ are phagocytosed by macrophages and dendritic cells, but do not induce apoptosis like NT _Salmonella_. Instead, typhoidal _Salmonella_ replicate within the phagocytes as they migrate to mesenteric lymph nodes where invasive/systemic infection is initiated. Figure modified from Urdaneta and Cassadesus, 2018 (49).

syndromes (50,51). The factors responsible for development of post-*Salmonella* infection sequela are currently unclear.

Enteric fever is initiated in a similar manner to NT-gastroenteritis. Infection begins at the intestinal mucosa where M cells, enterocytes, and dendritic cells internalize typhoidal *Salmonella* from the intestinal lumen. Unlike NT-gastroenteritis, typhoidal *Salmonella* invasion into the lamina propria is associated with minimal inflammation and the recruitment of mucosal macrophages and dendritic cells instead of neutrophils (32). The recruited macrophages and dendritic cells engulf the invading *Salmonella* but are unable to clear them as typhoidal *Salmonella* possess resistance to macrophage killing (52). Some typhoidal *Salmonella* remain and replicate within the antigen presenting cells, while others migrate to the mesenteric lymph nodes where they encounter additional macrophages and replicate further (53). A transient bacteremia develops as typhoidal *Salmonella* enter the circulatory system from the mesenteric lymph nodes and spread to organs throughout the host (14). Typhoidal *Salmonella* invade and incubate in multiple locations in the host, such as the spleen, liver, bone marrow, and gallbladder, for up to two weeks before clinical signs of infection are detected. Patients with typhoidal infection of the gallbladder may develop a chronic carrier state, where they serve as an asymptomatic reservoir for typhoidal *Salmonella* (38). Both enteric fever and chronic carrier state are resolved through antibiotic treatment, although multi-drug resistance strains have emerged (54).

1.1.4. *Salmonella* Epidemiology

The study of *Salmonella* disease distribution focuses on the subspecies *enterica,* where the majority of *Salmonella*-related infection stem from in humans. As such, *S.*

enterica epidemiology is divided into its two disease groups, typhoidal and NT *Salmonella*. NT *Salmonella*-related illness represents a significant burden to both developed and developing countries globally (55). Our current knowledge of NT *Salmonella* infection rates is largely reflective of industrialized nations as case reporting in many countries with poor health infrastructure is absent. In Canada and the United States, NT *Salmonella* ranks among the top three bacterial pathogens responsible for foodborne illness (56,57). Consumption and mishandling of foods of animal origin, especially chicken and raw eggs, are common risk factors associated with NT *Salmonella* infection (58). However, many outbreaks associated with NT *Salmonella* are linked to contaminated plant-based products. The largest NT *Salmonella*-related outbreaks in North American history were linked to contaminated peanut butter and contaminated cucumbers, resulting in 714 and 907 reported cases, respectively (59,60). Outbreaks generally contribute to a minority of NT *Salmonella* infections as most reported cases are related to endemic infection (i.e. transmission from natural reservoirs) (57). In Canada, nearly all endemic NT *Salmonella* infections are associated with gastroenteritis, whereas 15% of cases require hospitalization for additional complications. These infections are largely caused by *Salmonella* ser. Enteritidis (57), similar to the United States (61). While gastroenteritis is the primary complication of NT *Salmonella* cases, many studies have investigated the risk factors associated with the more severe, but less prevalent bacteremia.

The probability of acquiring NT-bacteremia in a developed country is relatively low compared to gastroenteritis. A study conducted by the International Bacteremia Surveillance Collaborative found a NT-bacteremia incidence rate of 0.81 per 100,000

when examining data from Finland, Canada, Australia, and Denmark (62). As of 2018, the incidence rates of NT *Salmonella* infection in Canada and the United States per 100,000 are 19.4 and 18.3, respectively (56,57). This relatively low incidence rate of NT-bacteremia does not accurately represent the susceptibility of specific at-risk populations to invasive NT *Salmonella* infection. Numerous studies have demonstrated that both neonates and the elderly are particularly at higher risk of developing bacteremia when infected with NT *Salmonella* (33,63,64). Individuals with underlying health conditions, such as cancer (65), lupus (66), and anemia (67), and the immunocompromised are also at increased risk of NT-bacteremia (68). In addition to host factors, specific NT *Salmonella* serovars, such as Dublin, Choleraesuis, and Virchow, appear to produce invasive disease at a higher frequency than others (63,64,69). A more recently discovered NT *Salmonella* subtype, sequence type 313 (ST313) of *Salmonella* ser. Typhimurium, is also closely associated NT-bacteremia, specifically producing invasive disease in immunocompromised patients in African countries (70). Why these serovars are particularly adept at causing bacteremia is not completely understood. Considering the numerous aspects that contribute to *Salmonella* disease progression, it is likely that a combination of host and pathogen factors are responsible.

While NT *Salmonella* is prevalent globally, typhoidal *Salmonella* is primarily endemic in developing countries. As of 2017, high incidence rates of typhoidal *Salmonella* infection were reported in Sub-Saharan and Northern Africa, South and South-East Asia, and the Middle East (71). These regions are often associated with inaccessibility to clean drinking water, poor sanitation infrastructure, and hygienic practices that promote transmission of enteric pathogens. Transmission of typhoidal

Salmonella is specifically high in these regions, and not in developed countries, because of the prevalence of acute and chronic carriers (38). Typhoidal *Salmonella* are completely reliant on carriers for the transmission of disease as humans are the only natural reservoir. Accordingly, transmission of typhoidal *Salmonella* is favourable in areas where sanitation practices allow for contamination of drinking water sources with human feces. Unlike NT *Salmonella*, typhoidal *Salmonella* infections are primarily observed in early ages, from newborns to young adults (72,73). Additionally, patient age and pathogen antimicrobial resistance are not considered prognostic of enteric fever mortality rates in endemic regions, which range between 1-3% (74). Most patients acutely shed typhoidal *Salmonella* following the clearance of infection. However, patients with a history of gallstones are at higher risk of developing a chronic carrier state as typhoidal *Salmonella* form biofilms on gallstones, increasing their resistance to clearance (75). Outside of endemic regions, cases of enteric fever are typically associated with travel to areas where typhoidal *Salmonella* infections are prevalent (76). However, cases associated with domestic transmission are still observed due the contamination of food products by chronic carriers (77).

1.2. *Salmonella* Infection Models

Salmonella research relies heavily on infection models to study *Salmonella* virulence and host-pathogen interactions. Researchers have developed a variety of infection models to study *Salmonella* due its broad host range and diversity in clinical manifestations. Presented here is a summary of the current infection models used to study *Salmonella* pathogenesis and its associated diseases in humans.

1.2.1. *In vitro* Mammalian Cell Models

Immortalized cell lines are widely used in the study *Salmonella* pathogenesis. Several different cell lines are needed to study *Salmonella* as *Salmonella* interact with a variety of cell types during infection. Each cell line has strengths and weaknesses as a model of *Salmonella* pathogenesis in humans. For instance, the COS-1 and MDCK cell lines, primate fibroblasts and canine epithelial cells, respectively, are susceptible to *Salmonella* infection and are useful for their genetic amenability but lack relevance to human infection due to their animal origin (78,79). Immortalized human cells such as the Hep-2, HeLa, HEK-293, and Int-407 cell lines are more commonly used in *Salmonella* infection models, and assist in the study of invasion, intracellular replication, and host signaling (80–82). However, these cell lines lack some biological relevance as they are derived from tissues only encountered during systemic *Salmonella* infection. To model human GI infection more closely, intestinal epithelial cells such as the T84, Caco-2, and HT-29 cell lines are typically used (83–85). These cell lines form polarized monolayers in culture with apical and basolateral surfaces resembling those observed in the human intestine. Additionally, T84 and Caco-2 cell lines differentiate in culture into enterocytes, a cell type *Salmonella* interacts with at the site of infection (86). The Caco-2 cell line is also used to generate an *in vitro* M cell model to study *Salmonella* translocation across the polarized monolayers of epithelial cells (87). In addition to intestinal epithelial cells, immortalized monocytes, such as the THP-1 and U937 cells lines, assist in modeling *Salmonella* infection in humans. Both THP-1 and U937 cell lines can be activated in culture to study *Salmonella* survival and virulence within macrophages (88,89), a critical interaction during disease progression. This interaction is also studied using the murine

macrophage cell lines J774A.1 and RAW264.7 (90,91). Although *in vitro* cell models provide insight into *Salmonella* infection in humans, the knowledge gained is largely cell-type and single cell specific.

Advancements in *in vitro* modelling of *Salmonella* infection in humans have transitioned from two-dimensional single-cell type culture to three-dimensional mixed-cell tissue culture. The first three-dimensional tissue culture infection model followed the introduction of a rotating-wall vessel to culture Int-407 and HT-29 cell lines into tissue aggregates (92–94). These aggregates were receptive to *Salmonella* infection but displayed less *Salmonella* invasion relative to monolayer infections. The addition of activated macrophages (U937 cells) to HT-29 tissue aggregates later improved the model's ability to distinguish between *Salmonella* strains varying in virulence (95). Further advancements in three-dimensional cell culture infection models involved the use of stem cells to generate intestinal organoids and enteroids. Intestinal organoids are derived from induced pluripotent stem cells which differentiate into a mixture of enterocytes, goblet cells, Paneth cells, and enteroendocrine cells, all of which are present in the intestinal epithelium. Enteroids, however, originate from intestinal stem cells and only contain epithelial cells (96). Although both intestinal organoids and enteroids possess cellular architecture that resembles the human intestine, organoids are considered more relevant to human infection because of their heterogeneity in cell types (97). Regardless, multiple studies have used either intestinal organoids or enteroids as models of *Salmonella* infection with success (98–102). A final advancement in three-dimensional cell culture follows a multi-layered approach to construct the human intestine *in vitro*. Schulte *et al* have created an infection model whereby layers of epithelial cells, collagen,

endothelial cells, and immune cells stack together to form a replica of the intestinal mucosa (103). While this model is currently limited to *Salmonella* invasion into the epithelial layer only, its construction represents significant progress in *in vitro* modeling of *Salmonella* infection in humans. Despite all these advancements in modelling human infection *in vitro*, cell culture infection models are still unable to provide valuable insight into *Salmonella* disease progression.

1.2.2. *In vivo* Infection Models

In vivo models of *Salmonella* infection permit the study of disease progression, an advantage that is absent in *in vitro* models. Several *in vivo* models are used to study *Salmonella* infection due to the broad host range and clinical manifestations of *S. enterica* subsp. *enterica* serovars. In particular, the oral calf infection model is valuable for both the study of *Salmonella*-associated gastroenteritis and bacteremia (104). This model produces enteric illness in calves that resembles *Salmonella* infection in humans in both clinical symptoms and pathological changes (45). The clinical manifestations vary based on virulence of the serovar and dosing, as calves given low oral doses of *Salmonella* Typhimurium typically exhibit transient diarrhea, while high doses are associated with bacteremia and lethal infection (105,106). This range in disease is especially useful in characterizing the virulence of *Salmonella* strains through clinical scoring and examining intestinal pathology (107). Although the *Salmonella* infection model in calves is an accurate representation of human disease, it is limited by costs and logistics associated with performing multiple experiments and genetic variability between animals (108). The introduction of a ligated ileal loop model in calves alleviated some of these limitations. Instead of infecting multiple animals, this model enables separate *Salmonella* infections

in individually ligated loops of a single ileum (107). Loop infections are typically restricted to the study of acute *Salmonella* infection (<24h), but alternations to the technique have permitted longer infection periods (5 days) (109). The ileum loop model measures *Salmonella* virulence through examining intestinal pathology and quantifying bacterial burdens in each loop (104). These ileum loops improve upon the oral calf model as they evaluate multiple *Salmonella* strains in the same animal simultaneously. While the success of this technique has also translated to rabbits (110,111) and pigs (112,113) as *in vivo* models of *Salmonella* infection, the model is still burdened by the animal housing logistics and the expertise required to perform the surgery.

The most prominent *in vivo* model used to study *Salmonella* infection is the streptomycin treated mouse model developed by Dietrich-Hardt and co-workers (114). In this model, mice are treated with the antibiotic streptomycin to remove gut microflora, which provide colonization resistance to *Salmonella* infection (115). Pretreated mice are then orally inoculated with *Salmonella* leading to intestinal inflammation coupled with epithelial lesions and endothelial infiltrate rich in polymorphonuclear leukocytes (116). These responses to *Salmonella* infection are similar to those observed in the calf infection model (109). However, diarrhea is not typically reported during infection in the streptomycin treated mice. Instead, fecal secretions appear slimy and pellet formation is absent (114). In addition to gastroenteritis, infected mice concurrently develop systemic disease akin to enteric fever in humans (114). These differences in clinical manifestations limit the utility of streptomycin treated mice as a model of *Salmonella* disease in humans. Regardless, experiments using pre-treated mice are favoured over calf infections as they are less labourious and are amendable to scaling. An additional advantage to the pre-

treated mouse model is the degree of control over both host and pathogen genetics. Multiple groups have used the streptomycin treated mouse model to screen *Salmonella* mutant libraries in order to identify important virulence genes (117–119), which requires numerous mice. Studies with immunodeficient mice have also used the streptomycin pre-treatment model to examine the impact of host genetics on *Salmonella* infection (120,121). While the streptomycin mouse model has its weaknesses, it remains a valuable tool is the study of NT *Salmonella* pathogenesis.

Most *in vivo Salmonella* models are valuable to the study of NT-gastroenteritis but fail to provide insight on host restricted conditions such as enteric fever. Considering enteric fever related to typhoidal *Salmonella* infection is restricted to humans, modelling a similar disease progression in animals is challenging. The current *in vivo* model used to study systemic *Salmonella* disease in animals is the murine infection model. In general, immunocompetent mice are susceptible to systemic infection with *Salmonella* ser. Typhimurium, characterized by an absence of gastroenteritis and invasion of *Salmonella* into the liver and spleen (53). There are multiple murine infection models that differ based on route of infection, genetic background of the mouse, and the *S. enterica* subsp. *enterica* serovar used for infection (122). Oral administration of *Salmonella* inoculum into mice is considered the most relevant inoculation method to human disease compared to intravenous (iv), intraperitoneal (ip), and subcutaneous injections, but requires a higher number of bacteria for successful infection (122). A large variance in susceptibility to infection is also observed between different mouse backgrounds with oral *Salmonella* ser. Typhimurium inoculation due to genetic differences in host immunity genes. Notable genetic variants are found within Slc11a1 (formerly Natural resistance-associated

macrophage protein 1, Nramp1) and Toll-like receptor 4 (TLR4) (123), which contribute to intramacrophage degradation of pathogens (124,125) and detection of LPS for activation of a pro-inflammatory immune response (126), respectively. Mouse backgrounds harboring these mutations have increased susceptibility to systemic *Salmonella* ser. Typhimurium infection (127,128). While *Salmonella* infection progresses similarly for both the immunocompetent murine model and enteric fever in humans (129), the serovars involved for each are quite different. *Salmonella* ser. Typhimurium is genetically distinct from typhoidal *Salmonella* serovars and is restricted to gastroenteritis during human infections (32), whereas typhoidal *Salmonella* do not establish infection in immunocompetent mice (130). Therefore, *Salmonella* ser. Typhimurium infection in mice represents an incomplete model of enteric fever in humans.

Another approach to modelling enteric fever in mice involves the humanized mouse model. In this model, human hematopoietic stem cells are engrafted into an immunodeficient mouse background to generate human immune cells. Following engraftment, humanized mice are susceptible to *Salmonella* ser. Typhi and exhibit innate and adaptive immune responses characterized by the production of human cytokines in response to infection (131). The inoculum is delivered either by ip or iv injection as oral administration of *Salmonella* ser. Typhi is incompatible with intestinal invasion in mice (132). Multiple groups have performed *Salmonella* ser. Typhi infections in humanize mouse models with varying results. Mouse survival differed in studies using ip administration of *Salmonella* ser. Typhi with one group reporting persistent non-lethal infections (133), while others observed acute fatal disease (134). Another group using iv inoculation reported high bacterial burdens in the liver, spleen, and bone marrow, along

with significant weight loss and high survival (135). Despite these differences in infection outcomes, multiple studies have screened *Salmonella* ser. Typhi transposon libraries through the humanized mouse model and have successfully identified genes important to systemic infection (134,136). These findings highlight the potential utility of humanized mouse as a model for systemic *Salmonella* infection in humans, although an absence of intestinal invasion still limits its relevancy.

In addition to the mammalian infection models discussed here, ectotherm models, such as *Caenorhabditis elegans* and zebrafish, are becoming increasingly more prominent in the study of *Salmonella* pathogenesis. Both the *C. elegans* and zebrafish models are susceptible to *Salmonella* infection and display conservation of host-pathogen interactions between *Salmonella* and mammalian models (137–139). These two ectotherm models have the innate benefit of transparent physiology permitting non-invasive microscopy to monitor fluorescent *Salmonella* growth and clearance *in vivo* during infection. *Salmonella* research involving these two ectotherm models has primarily focused on investigating host-pathogen factors contributing to bacterial persistence. Studies using *C. elegans* as a *Salmonella* infection model have characterized the function of virulence genes (140), evaluated the virulence of *Salmonella* pathotypes (141), and examined *in vivo* biofilm formation (142). *Salmonella* studies using the zebrafish model have confirmed important roles for macrophages and neutrophils in pathogen clearance (143) and have verified the importance of cholesterol in *Salmonella* disease outcomes (144). While these ectotherm models have contributed to the study of *Salmonella* pathogenesis, they are still limited by their inherent differences to mammalian infections. *Salmonella* infection in both *C. elegans* and zebrafish are performed at lower

temperatures (< 30°C) relative to mammalian models (137,139). likely influencing pathogen protein expression. They are also ectotherms, which are physiologically distinct from mammals and possess notably different immune systems (145,146). Nevertheless, the *C. elegans* and zebrafish models do hold several advantages. Both models are amendable to genetic manipulation, are scalable for replicates in high-throughput screenings, and are more favourable from an animal ethics perspective relative to mammalian models (104). For these reasons, both the *C. elegans* and zebrafish models show promise as low-maintenance screening models for *Salmonella* virulence.

1.2.3. Protozoan Infection Models

In the study of bacterial pathogenesis, bacterivorous protozoa are occasionally used as a supplement to macrophage experiments. Both protozoa and macrophages use similar mechanisms for phagocytosis of bacteria and degradation of phagosomal contents (147–149). The virulence factors used by intracellular bacteria to evade and resist consumption and digestion by macrophages often translate to resistance during protozoan grazing (150–152). A well characterized example of this versatile resistance is observed with *Legionella pneumophila* as they avoid degradation and replicate in both amoeba and macrophages (153–156). In the absence of macrophage infection modelling, protozoa can serve as a valuable replacement for the study of intracellular bacteria.

There are three main protozoan models that have been used to study the intracellular resistance mechanisms of *Salmonella*. The first model involves ciliates from genus *Tetrahymena*. *Tetrahymena* spp. are highly motile protists that are covered in cilia and are constantly sampling smaller particles from their environment. The cilia act collectively to facilitate protist movement and to sweep smaller particles into the oral

groove (157). Smaller particles, like bacteria, are phagocytosed in a food vacuole upon entering the oral groove and are subjected to digestion as a food source for *Tetrahymena* spp. (158). Unused components from digestion are then excreted through the anal pore as membraned vesicles known as fecal pellets (158). As a model for bacterial infection, *Tetrahymena* are typically co-cultured with bacteria in saline to assess uptake, food vacuole size, bacterial resistance to degradation, and ciliate viability (159,160). Studies evaluating *Salmonella* resistance in *Tetrahymena* spp. have demonstrated that *Salmonella* are resistant to degradation following phagocytosis and remain viable in food vacuoles and in fecal pellets after excretion (161). Microarray gene profiling performed on phagocytosed *Salmonella* indicates that many genes important to survival within macrophages are also upregulated when enclosed in *Tetrahymena* food vacuoles. Additionally, knocking out genes associated with acid tolerance in *Salmonella*, which are also important for resistance to macrophages, resulted in lower viability of phagocytosed bacteria (162). These studies do not report changes in *Tetrahymena* viability in response to *Salmonella* co-culture, although other work involving *E. coli* O157:H7, a shiga-toxin producing serotype, show decreases in ciliate counts following co-incubation (163).

A second protozoan model of *Salmonella* infection involves the amoeba *Dictyostelium discoideum* (hereafter *Dictyostelium*). *Dictyostelium* are primarily found in soil where they phagocytose and kill bacteria as a source of nutrients (164). When nutrient deprived, *Dictyostelium* collectively enter a sporulation cycle to hibernate until growth conditions are optimal (165). Entrance into the sporulation cycle from the unicellular stage is used as an indication of whether *Dictyostelium* are feeding, starving, or viable. Unlike *Tetrahymena, Dictyostelium* infections are performed both in liquid co-

culture and on agar plates. An agar infection assay involves plating *Dictyostelium* onto a bacterial lawn and monitoring the plate for spore formation. *Dictyostelium* co-culture experiments assess bacterial virulence through enumerating bacterial burdens and measuring amoeba viability (164). Studies using *Dictyostelium* co-culture experiments as a model of *Salmonella* infection have had varying results. *Dictyostelium* infections using a low multiplicity of infection (MOI) of 1:1-5 showed degradation of *Salmonella* after two days (166,167), while others showed intracellular replication when higher MOIs were used (1:100-1000)(168,169). Results from agar plating experiments demonstrate that *Salmonella* halts *Dictyostelium* sporulation and decreases amoeba viability (166). Despite this variability in *Salmonella* degradation, numerous groups have identified *Salmonella* genes important to resistance in *Dictyostelium* that are known contributors to intracellular growth in mammalian models (169–171). The *Dictyostelium* model can also evaluate the contributions of host genes to *Salmonella* clearance as the amoeba possess similar gene homology to humans, such as with Slc11a1 and autophagy machinery (172), and are genetically amendable to mutations (164). The ability to manipulate both host and pathogen genetics with *Dictyostelium* infection make it a useful tool for the study of *Salmonella* virulence.

The final protozoan model used to study *Salmonella* infection involves amoeba from the genus *Acanthamoeba*. *Acanthamoeba* spp. inhabit several different environments and are isolated from a variety of sources including soil, plants, fresh water, sea water, and sewage (173). In these environments, *Acanthamoeba* spp. ingest and degrade bacteria for nutrients in a similar manner to *Dictyostelium*, but form stress resistance cysts under nutrient poor conditions instead of spores (174). In a laboratory

setting, *Acanthamoeba* spp. behave more closely to mammalian macrophages than *Dictyostelium* as they adhere to surfaces when in axenic culture. Accordingly, experiments investigating bacterial virulence in *Acanthamoeba* spp. are conducted similarly to infections in mammalian cell *in vitro* models (175–177). Studies using *Acanthamoeba* spp. as a model of *Salmonella* infection have varying results on bacterial clearance. Multiple studies demonstrate that *Salmonella* are degraded by *Acanthamoeba* spp. (176,178,179), while others report intracellular replication over the course of infection (180–182). Although the MOIs used in all these studies differed greatly (between 10 and 1000), the fate of *Salmonella* in *Acanthamoeba* spp. appears to be independent of the inoculum to amoeba ratio. What does impact bacterial clearance in *Acanthamoeba* spp. is *Salmonella* virulence. A study examining the contributions of *Salmonella* virulence factors to *Acanthamoeba* spp. digestion resistance found that these factors are important for intracellular survival in amoeba, similar to their function in mammalian macrophages (181). Aside from this study, there is minimal research investigating the impact of specific *Salmonella* virulence factors on survival in *Acanthamoeba* spp. From a host standpoint in the *Acanthamoeba* spp. model, amoeba viability correlated with MOI in response to *Salmonella* infection. Infecting with higher amounts of *Salmonella* was associated with amoeba apoptosis (182), whereas *Acanthamoeba* spp. levels remained stable or increased with lower inoculums (178,179). Altogether, the amoeba viability and bacterial clearance phenotypes associated with *Acanthamoeba* spp. paired with their low maintenance cost make them a useful model for the study of *Salmonella*.

1.3. *Salmonella* Molecular Pathogenesis

Salmonella pathogenesis is conferred by a range of virulence factors. These virulence factors are spread across various genomic islands in the *Salmonella* genome. Some of these genomic islands and virulence factors are conserved throughout subspecies of *Salmonella*, while others are encoded by specific serovars. Presented here is a summary of the most notable genomic islands in *Salmonella*, their virulence factors, and how they contribute to pathogenesis.

1.3.1. *Salmonella* Pathogenicity Islands

Genomic islands encoding genes associated with bacterial pathogenesis are commonly referred to as pathogenicity islands. In *Salmonella*, pathogenicity islands regulate many of the processes important to survival within a host, such as host cell invasion, immune evasion, and intracellular replication (183). Several of these processes are shared among *Salmonella* serovars as SPIs are mobile genetic elements that move between strains via horizontal gene transfer (184). Many SPIs are differentially distributed among *Salmonella* subspecies and serovars because of this genetic movement. The acquisition of specific SPIs coincide with evolutionary events that define *Salmonella* taxonomy (Figure 1.1B). For example, the genus *Salmonella* diverged from a common *Enterobacteriaceae* ancestor with *E. coli* after acquiring SPI-1 (185). The speciation of *Salmonella* is also linked to horizontal gene transfer as *S. enterica* diverged from *S. bongori* after acquiring SPI-2 (20). An initial complete genome sequence of *Salmonella* revealed a total of ten SPIs (SPI-1 – SPI-10) (186). The total number of SPIs continues to increase with SPI-23 being the latest identified (187). The discovery of new SPIs is not necessarily applicable to all serovars, however, as there is variability in the conservation

of pathogenicity islands (188). While many SPIs are considered variable in their serovar distribution, SPI-1 and SPI-2 are highly conserved (189), and both play instrumental roles in *Salmonella* pathogenesis.

1.3.1.1. *Salmonella* Pathogenicity Island-1

SPI-1 is a 40 kb genomic island that encodes conserved 39 genes (Figure 1.3), a majority of which contribute to epithelial cell invasion (190). These genes encode transcription factors, chaperones, type three secretion system (T3SS) components, and secreted proteins referred to as effectors. When expressed, SPI-1 genes work in concert to facilitate internalization into non-phagocytic epithelial cells (191). Expression of these genes is influenced by various environmental factors such as oxygen concentration, osmolarity, pH, Mg^{2+}, bile and short-chain fatty acids, most of which are associated with the conditions of the GI tract (192). These environmental stimuli regulate expression of three AraC-like transcription factors of the SPI-1 hyperinvasion locus D (*hilD*) and *hilC*, and *rtsA*, which is located outside the pathogenicity island (193). Together, these three transcription factors form a complex feed-forward loop involving hetero- and homodimers to upregulate expression of the SPI-1 master regulator *hilA* and a secondary SPI-1 regulator *invF* (194). Both HilA and invasion protein F (InvF) bind to promoters within and outside the SPI-1 locus to activate expression of SPI-1 T3SS components, chaperones, and effectors to prime the bacterium for epithelial cell invasion (195).

Upon contacting an intestinal epithelial cell, the surface expressed SPI-1 T3SS (T3SS-1) of *Salmonella* penetrates the plasma membrane through insertion of the *Salmonella* invasion protein (Sip) translocon pore, SipB-SipC (196). Insertion of SipC
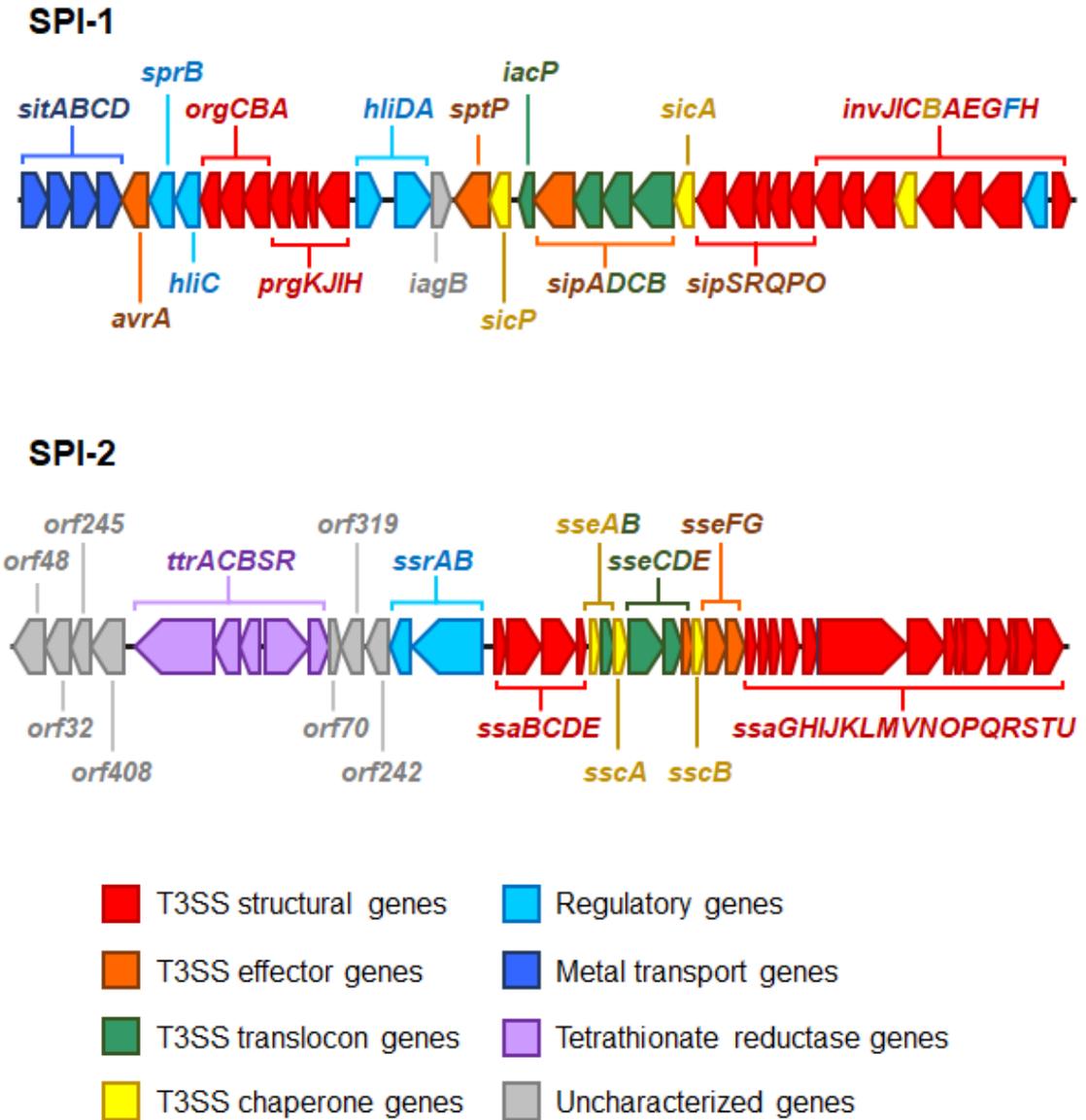
Figure 1.3: Genes of *Salmonella* pathogenicity islands 1 and 2.
Conserved genes found in the 40 kb pathogenicity islands SPI-1 and SPI-2 of *S. enterica* are illustrated above. Genes are coloured based on function of the encoded protein. Modified from Hurley et al, 2014 (197).

into the plasma membrane immediately disrupts actin dynamics at the cytosolic surface through actin nucleation and bundling (198,199). Following translocon insertion, the T3SS-1 translocates a range of effectors to further destabilize host cytoskeletal architecture to induce phagocytosis of the bacterium. The effector SipA binds directly to actin at the site of invasion to stabilize actin filaments and to induce cytoskeletal rearrangement and membrane ruffling, assisting in phagocytosis (200–202). Three additional effectors encoded outside the SPI-1 locus, *Salmonella* outer protein B (SopB), SopE, and SopE2, contribute to *Salmonella* invasion via activation of host Rho-Guanosine triphosphophatase (Rho-GTPases). Both SopE and SopE2 function as guanine nucleotide exchange factors (GEFs) and activate Rho-GTPases by facilitating the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) (203–208). SopB activates additional Rho-GTPases through remodeling the phosphatidylinositol phosphate (PIP) landscape via its phosphatase activity at the plasma membrane (209–211). Together, SopB, SopE, and SopE2 activate several host Rho-GTPases to facilitate actin polymerization and actomyosin-mediated contractility at the site of secretion (212–216).

In addition to facilitating invasion, several SPI-1 effectors activate host signaling pathways leading to cytokine expression. For example, Rho-GTPase activation linked to SopE, SopE2, and SopB activates NF-κB, initiating a pro-inflammatory response (217). SipA activity is also associated with stimulating IL-8 expression and recruitment of neutrophils through a similar pathway (218–220). Additionally, these four effectors (SopE, SopE2, SopB, and SipA) disrupt tight junctions in epithelial cells and compromise the barrier function of the intestinal epithelium (221). Invasion-associated inflammation

is also initiated with activation of cysteine-dependent aspartate-directed protease 1 (caspase-1) and caspase-3. Activation of caspase-1 is linked to host recognition of the T3SS-1 components PrgJ and SipB (222–225), as well the GEF activity of SopE (226,227), while caspase-3 is activated in response to SipA secretion in macrophages (228). Three T3SS-1 effectors, avirulence protein A (AvrA), SopA, and *Salmonella* protein tyrosine phosphatase A (SptA), counteract these consequences of *Salmonella* invasion through inhibition of host signaling pathways. AvrA, an acetyltransferase, inhibits signaling through the c-Jun N-terminal kinase (JNK) pathway and, as a result, dampens the inflammatory response (229), impairs apoptotic signaling in the infected cell, (230) and strengthens tight junctions in the intestinal epithelium (231). SopA, a homologous to the E6-AP carboxyl terminus (HECT)-like E3 ubiquitin ligase, is also associated with counter-balancing the inflammation elicited by T3SS-1 secretion effectors (232). Specifically, SopA ubiquitinates tripartite motif (TRIM) 56 and TRIM 65 resulting in their degradation and subsequent suppression of interferon-β (IFN-β) (233). SptP, a dual function effector, suppresses inflammation through its GTPase activating protein (GAP) domain and a tyrosine phosphatase domain. The GAP activity of SptP is associated with reversing Rho-GTPase activation linked to the GEF activity of SopE and SopE2 and preventing downstream signaling of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) (234,235), while the tyrosine phosphatase activity dephosphorylates Raf in the extracellular signal-regulated kinase (ERK) pathway dampening expression of pro-inflammatory cytokines (236,237). SptP also plays a role in invasion through dephosphorylation of the host enzyme villin, which regulates actin dynamics at the cytosolic surface during *Salmonella* internalization (238).

Following internalization into intestinal epithelial cells, *Salmonella* are enclosed into phagosomes, or what are commonly referred to as *Salmonella* containing vacuoles (SCV). Although SPI-1 expression is downregulated in *Salmonella* within the SCV (239), T3SS-1 effectors continue to manipulate host processes for infection. The T3SS-1 effectors SopB and SopD facilitate membrane fission after phagocytosis leading to the formation of the SCV (240). In addition to membrane fission, SopB contributes to survival of *Salmonella* within the SCV. The phosphatase activity of SopB at the plasma membrane is indirectly associated with activation of the anti-apoptotic signaling kinase protein kinase B (Akt), which extends host cell life for *Salmonella* intracellular replication (241–243). Additionally, SopB remodels the PIP landscape at the surface of the SCV for selective recruitment of Rab guanosine triphosphatases (Rab-GTPases) (244,245), Rho-GTPases (211), and sorting nexins (SNX) (246) to inhibit lysosome fusion and promote intracellular replication. Intracellular survival within the SCV is also regulated by three additional SPI-1 effectors, SipA, SptP, and SopF. SipA mimics a soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) to reroute syntaxin 8 from the host vesicle trafficking pathway to the SCV where it promotes replication (247). SptP also promotes intracellular survival through dephosphorylating the host adenosine triphosphatase (ATPase) p97, which increases membrane stability of the SCV (248). Similarly, the recently identified T3SS-1 effector SopF localizes to the SCV and is required to maintain nascent SCV membrane integrity (249,250). SopF possesses ADP-ribosyltransferase activity that is responsible for modifying SCV-associated V-ATPases and blocking bacterial clearance via xenophagy

(251). Whether this ADP-ribosyltransferase activity also contributes to SCV stability is currently unknown.

Although intracellular replication of *Salmonella* is typically associated with the SCV, a portion of internalized *Salmonella* escape form the phagosome into the cytosol where they exhibit a hyper replication phenotype (252). This escape from the phagosome is regulated by many virulence factors. Recently, Stévenin et al. demonstrated that SopB contributes to phagosome escape through modulating SCV size in response to macropinosome formation during invasion (253). Upon entering the host cytosol, SPI-1 expression is upregulated in response to the environmental conditions (254). As a result, a second wave of T3SS-1 effectors are secreted into the cytosol in a SPI-1 T3SS transolocon-independent process (255). Second wave secretion of both SopB and SipA is required for *Salmonella* survival within the host cytosol. The second wave of SopB secretion induces a second increase in Akt signaling which prolongs host cell survival and increases the duration of intracellular replication (256), while secretion of SipA impairs autophagic targeting of cytosolic *Salmonella* preventing their degradation (257). The secretion of other T3SS-1 effectors such as SopE, SopE2, SipA, and SopD in *Salmonella* does not impact cytosolic replication (256).

1.3.1.2.*Salmonella* Pathogenicity Island-2

SPI-2 is a 40 kb genomic island that contains 43 conserved opening reading frames (Figure 1.3) (258,259). Holden and colleagues identified SPI-2 through performing a signature-tagged mutagenesis screening of *Salmonella* through a systemic mouse infection model (260). The virulence genes identified from the screening were similar to SPI-1, but functionally distinct. Instead of contributing to invasion, the SPI-2

locus promotes intracellular survival of *Salmonella* (261). The SPI-2 locus encodes transcription factors, chaperones, secretion effectors, and components of a T3SS separate from the system located in SPI-1 (259). A smaller portion of SPI-2 also encodes a tetrathionate reduction system that provide a nutritional fitness advantage over gut commensals during inflammation (262) and other uncharacterized open-reading frames (ORFs) that do not contribute to virulence (263). Expression of SPI-2 virulence factors coincides with the environmental conditions associated with the SCV including low pH, iron, phosphate, and Mg^{2+} levels (264–268). These conditions activate the SPI-2 two component system *Salmonella* secretion regulator AB (SsrAB), which induces expression of the SPI-2 T3SS (T3SS-2) as well as effectors that are encoded within SPI-2 and outside the pathogenicity island (269). When the SPI-2 locus is expressed, the effectors, chaperones and T3SS-2 work collectively to establish an intracellular niche for *Salmonella* replication.

Formation of the *Salmonella* intracellular niche is dependent upon effector modulation of the host endosome trafficking pathway. Diversion of the endosome pathway is important for maintaining membrane stability of the SCV and for nutrient delivery required for intracellular replication (270). Endosome delivery to the SCV also stimulates the formation of vesicular protrusions commonly referred to as *Salmonella* induced filaments (SIFs) (271). SIFs form a complex, interconnected network of tubules that originate from the SCV and extend throughout the cell where they acquire endosomal content (272). This SIF network functions as a pipeline for the efficient delivery of intravacuolar nutrients required for the maturation of the SCV and intracellular replication of *Salmonella* (273). The T3SS-2 effector *Salmonella* induced

filament protein A (SifA) is traditionally viewed as the major contributor to SIF formation, although several other effectors are implicated in tubule biogenesis (274–276). SifA primarily contributes to SIF formation through modulating host trafficking pathways via its interactions with late endosome/lysosome adaptor proteins. Interactions with Pleckstrin homology domain-containing family M member 1 (PLEKHM1) and PLEKH2M facilitate SifA-dependent delivery of the homotypic fusion and protein sorting (HOPS) complex to the SCV with late endosomes/lysosomes as cargo (277,278). The delivery of this cargo involves the motor protein kinesin-1 which is regulated by the SifA and T3SS-2 effector pathogenicity island encoded protein B2 (PipB2) at the SCV (279,280). SifA also binds Hermansky–Pudlak syndrome 3 protein (HPS3), a component of biogenesis of lysosome-related organelles complex 2 (BLOC-2), to deliver lysosomal-associated membrane protein 1 (LAMP1) positive lysosomes to the SCV (281). The lysosomes delivered to the SCV have reduced hydrolytic activity as SifA interferes with the mannose-6 phosphate receptor (MPR) recycling, leading to misrouted secretion of lysosomal enzymes (282). SifA-dependent delivery of these late endosomes/lysosomes maintains SCV membrane stability, regulates SIF formation, and promotes intracellular replication during infection. How SifA specifically modulates late endosome/lysosome adaptor proteins remains to be determined. The crystal structure of SifA shares similarity to the T3SS-1 effector SopE, suggesting SifA may function as a GEF (283); however, this GEF activity has not been confirmed.

In addition to SifA, several other T3SS-2 effectors contribute to SIF biogenesis. These effectors include *Salmonella* secretion effector F (SseF) and SseG which are inserted into the SCV as integral membrane proteins upon secretion (284,285). Both SseF

and SseG bind acyl-CoA binding domain containing 3 (ACBD3), a multifunctional cytosolic Golgi network-association protein, and tether the SCV to the Golgi network (286). This tethering restricts the SCV to perinuclear positioning within the host cell and promotes intracellular replication of *Salmonella*. The absence of SseF and SseG during *Salmonella* infection is associated with dispersed SCV position, reduced intracellular replication, and diminished SIF formation (284,287). SCV positioning and SIF formation is also linked to the T3SS-2 secreted protein *Salmonella* translocated effector A (SteA). During infection, SteA localizes to SIFs and the SCV in a phosphatidylinositol 4-phosphate (PI(4)P) dependent manner (288). Without SteA secretion, SIF formation is reduced and SCVs display abnormal morphology with microclustering of intracellular *Salmonella*. Additionally, deletion of *steA* in a *sseF* or *sseG* mutant exhibits dispersed SCV positioning during infection similar to that observed in the *sseF sseG* double mutant (289). While the exact function of SteA is unknown, these observations suggest that the SPI-2 effector regulates SCV membrane dynamics during infection.

SIF formation and SCV maturation are also regulated by the T3SS-2 effectors SopD2 and SseJ. *Salmonella* infections lacking either effector are associated with a reduction in SIF formation (290,291). Additionally, the secretion of SopD2 and SseJ antagonize the activity of SifA as they promote membrane instability when SifA is absent (292,293). Despite the similarities in SIF and SCV phenotypes, SopD2 and SseJ contribute to *Salmonella* infection in differently. SopD2 function as a GAP and binds several host small GTPases. As a GAP, SopD2 prevents the activation of GTPases subsequently impairing their activity in host endosomal trafficking (294,295). SopD2 effectively reduces endosome delivery to the SCV through its GAP activity, thereby

contributing to membrane instability and antagonizing SifA activity (296). The impact of SseJ on SCV membrane modulation is attributed to its acyltransferase function. Following secretion, SseJ localizes to the SCV where it is activated by RhoA (283). Activated SseJ esterifies cholesterol from the SCV membrane via its acyltransferase activity, transforming membrane cholesterol into cytosolic lipid droplets (297). The esterification activity of SseJ reduces cellular cholesterol leading to an increase in membrane rigidity, thus impacting SCV stability (298).

Aside from its acyltransferase activity, SseJ recruits and binds oxysterol binding protein 1 (OSBP1) to the SCV in conjunction with T3SS-2 effector SseL (299,300). OSBP1 is a lipid transporter that facilitates that transfer of cholesterol and PIPs between membranes (301). Knockdown of OSBP1 or deletion of both *sseL* and *sseJ* is associated with a reduction in SCV stability during *Salmonella* infection (300). The absence of OSBP1 also impairs intracellular replication of *Salmonella* (299). While SseJ may increase membrane rigidity through cholesterol esterification, it also manages to increase SCV stability via recruitment of OSBP1. The second T3SS-2 effector involved in OSBP1 recruitment, SseL, carries deubiquitinase (DUB) activity that is associated with regulating the lipid droplet formation during *Salmonella* infection (302). The DUB activity of SseL is also associated with inhibiting autophagic flux and increasing intracellular replication in infected macrophages (303). Previous work has demonstrated that SseL contributes to macrophage apoptosis and increased bacterial burdens during *in vivo* mouse infections (304,305). Whether these phenotypes of improved survival are attributed to SseL-associated OSBP1 recruitment or inhibition of autophagy remains to be determined.

Several other T3SS-2 effectors modulate host processes that are not associated

with SIF formation or SCV stability. For example, the effector SteC, which resembles the eukaryotic kinase Raf-1, stimulates extensive re-modelling of the filamentous actin (F-actin) cytoskeleton generating an F-actin meshwork surrounding SCVs (306). The F-actin remodeling is associated with SteC-dependent activation of the mitogen-activated protein kinase (MAPK) pathway involving Myosin IIB (307). How this F-actin meshwork contributes to *Salmonella* infection remains ambiguous as infection with a *steC* mutant displays a slight increase in intracellular replication and does not impact SCV stability (307). The similarly named T3SS-2 effector SteD contributes to *Salmonella* pathogenesis via inhibition of adaptive immunity. Following secretion, SteD localizes to the Golgi network and Golgi-derived vesicles as an integral membrane protein where it co-opts the host E3 ubiquitin ligase WW domain-containing protein 2 (WWP2) and the transmembrane protein 127 (TMEM127) to modify the mature major histocompatibility complex II (mMHCII) (308). Surface mMHCII levels are diminished in a SteD-dependent manner in antigen presenting cells, which suppresses T-cell activation (309). Thus, SteD represents an important virulence factor against the development of host adaptive immunity.

1.3.1.3. Remaining *Salmonellla* Pathogenicity Islands

There are currently 23 SPIs identified across the genus *Salmonella*. Research on SPIs has primarily focused on characterizing the contributions of SPI-1 and SPI-2 to *Salmonella* pathogenesis as they are present in nearly all *Salmonella* serovars that infect humans (189). The remaining SPIs associated with genus differ in their distributions and conservations between the various *Salmonella* species, subspecies, and serovars (188). As a result, how each of these SPIs contribute to *Salmonella* pathogenesis is better

understood for some more than others. Most SPIs are evaluated based on the proteins they encode and their contributions to *in vitro* infections and *in vivo* fitness. A brief review of the findings from studies involving SPIs 3-23 is provided below. A complete list of all SPIs and their ascribed functions during *Salmonella* infection is found in Table 1.1.

SPI-3 is a 17 kb genomic island that encodes ten genes, three of which are associated with *Salmonella* pathogenesis (310). Two of these genes are encoded in the *mgtCB* operon and contribute to intracellular survival in macrophages. Magnesium transport protein B (MgtB) functions as a magnesium transporter that regulates intracellular Mg^{2+} (311), while MgtC is involved in controlling membrane potential and promoting phosphate uptake in the SCV (312,313). The third gene, *misL*, encodes a type five secretion system (T5SS) that is involved in adhesion and biofilm formation and is associated with increased intestinal persistence *in vivo* (314). While SPI-3 is found across the *S. enterica* subspecies *enterica*, its genetic conservation is variable between serovars (188). Conversely, the 27kb genomic island SPI-4 is highly conserved across most serovars (315,316). SPI-4 encodes a type one secretion system and the giant adhesin *Salmonella* intestinal infection protein E (SiiE), which binds to the intestinal epithelial receptor MUC1 to facilitate *Salmonella* invasion (317,318). The expression of SPI-4 coincides with SPI-1 induction as the master regulator of SPI-1, HilA, is also responsible for inducing SPI-4 (319). Similar to SPI-4, SPI-5 is highly conserved across *Salmonella* serovars (188). The 7 kb genomic island encodes the T3SS-1 effector SopB and its chaperone PipC, as well as three T3SS-2 effectors PipA, PipB, and PipD (320). Although

Table 1.1: *Salmonella* pathogenicity islands

SPI	Function	Reference
SPI-1	Invasion into non-phagocytic cells	(191)
SPI-2	Intracellular survival	(321)
SPI-3	Intramacrophage survival	(310)
	Mg^{2+} transporter – *mgtB*	(311)
	Regulation of phosphate uptake and membrane potential – *mgtC*	(312,313)
	Type five secretion system (T5SS) and adhesion – *misL*	(314)
SPI-4	Co-regulated with SPI-1	(319)
	Type one secretion system (T1SS) - *siiABCDF*	(317)
	Adhesin – *siiE*	(318)
SPI-5	Required for enteritis in bovine ileal-loop model	(320)
	T3SS-1 effector and its chaperone – *sopB* and *pipC*, respectively	(322)
	T3SS-2 effectors – *pipA*, *pipB*, *pipD*	(323)
SPI-6	Chaperone-usher fimbriae operon - *safABCD*	(324)
	Outer membrane adhesin and invasin - *pagN*	(325,326)
	Type six secretion system (T6SS)	(327,328)
	Chaperone-usher fimbriae operon – *tcfABCD* (ser. Typhi)	(329,330)
SPI-7	Vi capsule biosynthesis locus – *viaB* operon	(82,331)
	Type four fimbriae locus – *pil* operon	(332,333)
	Found in serovars Typhi, Paratyphi, and some Dublin strains	(331,334)
SPI-8	Found serovar Typhi, inessential for intramacrophage survival	(335)
	Bacteriocin pseudogenes	(186)
SPI-9	Intestinal persistence	(336)
	T1SS and adhesin – *bapBCD* and *bapA*, respectively	(337,338)
SPI-10	Full and incomplete fimbriae operons – *sef* and *pef*, respectively	(339)
	Found in *Salmonella* ser. Typhi	
	Ser/Thr kinase and phosphatases – *prpZ* and *prkYX*, respectively	(340)
SPI-11	Intramacrophage survival – *pagC*, *pagD*, *mgsA*, *roaN, sopF*	(250,341,342)
	Typhoid toxin in *Salmonella* ser. Typhi – *pltA* and *pltB*	(343)
SPI-12	*In vivo* fitness in mice	(344)
	T3SS-2 effector E3 ubiquitin ligase – *sspH2*	
	O-antigen acetylate-transferase - *oafA*	(345)
SPI-13	Systemic pathogenesis in mice, upregulated in macrophages	(346,347)
	D-glucuronic acid and tyramine nutritional fitness	(348)
	Phagosome acidification and SPI-2 regulation – *lgl-ripABC*	(349)

SPI	Function	Reference
SPI-14	Upregulated in macrophages, putative electron transport proteins	(347,350)
	SPI-1 regulator in low O_2 - *loiA*	(351,352)
SPI-15	Found in Typhi, an integrase and four hypothetical proteins	(353)
SPI-16	Serotype conversion of LPS, highly homologous to SPI-17	(353,354)
SPI-17	Highly homologous to SPI-16, predicted to modify LPS	(353)
SPI-18	Found in Typhi and Paratyphi, few other *enterica* serovars	(355,356)
	Cytolysin that contributes to systemic infection in mice – *hylE*	(355)
	Promotes invasion into macrophages - *taiA*	(357)
SPI-19	T6SS, contributes to intracellular survival in murine macrophages	(358,359)
	Increased *Salmonella* ser. Gallinarum colonization in chickens	(360)
SPI-20	T6SS, found in *Salmonella enterica* subspecies *arizonae*	(358)
SPI-21	T6SS, found in *S. enterica* subspecies *arizonae*	(358)
SPI-22	T6SS, found in *Salmonella bongori*	(20)
SPI-23	Mainly found in serovars associated with livestock	(187)
	Contributes to adherence, regulation of surface pili - *potR*	(361)

the contributions of SopB to infection are well characterized, the impact of some of the remaining SPI-5 effectors on *Salmonella* pathogenesis is less defined. Neither *pipB* nor *pipC* have ascribed functions, but their absence during infection in a bovine ileal-loop model suppresses enteritis (320). Conversely, *pipA* encodes a redundant protease that cleaves RelA and RelB impairing NF-κB signaling, and subsequently inflammation (362).

In *Salmonella* ser. Typhimurium, SPI-6 spans 47 kb and encodes chaperone-usher fimbria within the *Salmonella* atypical fimbriae (*saf)* operon and an adhesin under *pagN*, both of which contribute to *Salmonella* adherence (324–326,363). SPI-6 also encodes a type six secretion system (T6SS) that enhances intracellular replication within macrophages and colonization in the mouse gut during *Salmonella* infection (327,328). In *Salmonella* ser. Typhi, SPI-6 contains an additional 8 kb insert including the Typhi

colonizing factor (*tcf*) cluster that encodes another chaperone-usher fimbria used in adherence (329,330). *Salmonella* ser. Typhi also encodes the 137 kb genomic island SPI-7, which in sparsely distributed in other serovars (364). SPI-7 contains a type IVb pilus used for invasion into epithelial cells and the Vi antigen B (*viaB)* operon responsible for the Vi exopolysaccharide capsule (82,332). While SPI-7 is mostly found in typhoidal *Salmonella*, it is also encoded and functional in some *Salmonella* ser. Dublin strains (334). The T3SS-1 effector SopE is also encoded within SPI-7 in *Salmonella* ser. Typhi but is located elsewhere on the chromosome in other serovars (331). Similar to SPI-7, SPI-8 is mainly found in *Salmonella* ser. Typhi (365). The 6.8 kb genomic island contains two bacteriocin pseudogenes and does not contribute to infection in macrophages (186,335).

SPI-9 is a 16 kb genomic island that is highly conserved across *S. enterica* subspecies *enterica* serovars (186,188). The biofilm associated protein (*bap*) operon found in SPI-9 encodes a T1SS and adhesin that contribute to biofilm formation and intestinal persistence during *in vivo* mouse infections (336). Unlike SPI-9, SPI-10 possesses high variability in both conservation and length between *Salmonella* serovars (339). In *S. enterica* ser Typhi, SPI-10 spans 33 kb and encodes an incomplete version of the *Salmonella* Enteritidis fimbriae (*sef)* operon and the protein phosphatase Z (*prpZ*) gene cluster, the latter of which enhances intracellular survival in macrophages (340). These SPI-10 associated elements are absent in *Salmonella* ser. Typhimurium (339). Both SPI-11 and SPI-12 were identified simultaneously from the whole genome sequence of *Salmonella* ser. Choleraesuis and display conservation and size variability across serovars (188,366). In *Salmonella* ser. Typhimurium, SPI-11 enhances intracellular survival

within macrophages (250,341,342), while in *Salmonella* ser. Typhi it encodes the typhoid toxin (343). Similarly, SPI-12 in *Salmonella* ser. Typhimurium enhances *in vivo* survival in mice and encodes the T3SS-2 effector *Salmonella* secreted protein H2 (SspH2), an E3 ubiquitin ligase, and the O-antigen acetyltransferase OafA (344,345).

SPIs 13 and 14 are variable genomic islands that were originally identified from the poultry restricted *Salmonella* ser. Gallinarum (367). Both SPIs are highly transcribed during *Salmonella* ser. Typhimurium infection in macrophages and contain genes that contribute to *Salmonella* pathogenesis (347). In *Salmonella* ser. Enteritidis, SPI-13 enhances nutritional fitness and infection kinetics during *in vivo* mouse infection (348). Additionally, the SPI-13 lactoylglutathione lyase *lgl* regulates SCV pH levels controlling the secretion of T3SS-2 effectors(349). SPI-14 encodes the transcription factor low oxygen induced factor A (LoiA) that upregulates *hilD* and *hilA* in low O_2 levels leading to increased expression of SPI-1 (352). This additional regulation of SPI-1 is absent in typhoidal *Salmonella* as SPI-14 is not found in the Typhi and Paratyphi serovars (351).

SPIs 15, 16, and 17 were identified simultaneously via bioinformatics in *Salmonella* ser. Typhi (353). These SPIs vary in function and distribution with SPIs 16 and 17 found in numerous serovars, while SPI-15 is present only in *Salmonella* ser. Typhi. The 6.5 kb genomic island SPI-15 contains an integrase gene as well as four hypothetical proteins with no ascribed functions (353). SPI-16 spans 4.5 kb and encodes genes that contribute to O-antigen glycosylation, which enhance *Salmonella* intestinal persistence during *in vivo* mouse infections (354). The 5.1 kb genomic island SPI-17 is highly homologous to SPI-16 and predicted to function similar manner (353). A small genomic island (2.3 kb) designated as SPI-18 is present in the *Salmonella* serovar Typhi,

but absent in many NT *Salmonella* serovars (355,356). SPI-18 encodes hemolysin E (HylE) and the secreted protein Typhi-associated invasion A (TaiA), the latter of which enhances uptake by macrophages (357).

The remaining SPIs (19-23) are sparsely distributed and mainly found in serovars that infrequently cause disease in humans. SPIs 19-22 were identified through comparative genomics where each genomic island was found to encode a T6SS (358). In *Salmonella* ser. Gallinarum, the SPI-19 T6SS enhances intracellular survival in murine and avian macrophages and colonization in an *in vivo* chicken model (360,368). Unlike the other SPIs, SPIs 20 and 21 are both solely present in the *S. enterica* subspecies *arizonae* (358), while SPI-22 is only found in *S. bongori* (20). Conversely, SPI-23 is found in several animal-adapted serovars and was also discovered via a comparative genomics approach (187). SPI-23 varies in size between 27-35 kb and encodes several putative T3SS effectors as well as the *pilV*-like gene *potR*, which is associated with increased adherence and invasion in pigs (361).

1.3.2. *Salmonella* Virulence Plasmids

Several serovars in the *enterica* subspecies possess a serovar-specific virulence plasmid that contributes to *Salmonella* pathogenesis. Although the plasmid for each serovar varies in size and genetic composition, they all encode a group of conserved virulence factors (369,370). These virulence factors are found in the *Salmonella* plasmid virulence (*spv*) operon (*spvRABCD*) and are associated with enhancing intracellular replication and systemic dissemination of *Salmonella* (371). Similar to SPI-2, the *spv* operon is induced in the intracellular environment of macrophages (372–374). Following induction, the transcription activator SpvR positively regulates *spv* operon expression

through initiating a feed-forward loop via binding both the *spvR* and *spvA* promoters (375). This SpvR-initiated induction is tightly regulated by the expression of SpvA, which quickly represses transcription of the *spv* operon (376). The result of this SpvR-SpvA co-regulation is a short-lived burst of expression from the *spv* operon.

The remaining genes expressed from the *spv* operon encode T3SS-2 effectors that contribute to *Salmonella* pathogenesis. For instance, *spvB* encodes an adenosine diphosphate (ADP)-ribose transferase that modifies globular actin (G-actin) following secretion, impairing actin polymerization (377). The ADP-ribosylation activity of SpvB is also associated with cytotoxicity in macrophages during infection, marked by the activation of caspase-3 (378). The second *spv* effector, SpvC, functions as a phosphothreonine lyase to dephosphorylate the MAPKs ERK, p38, and JNK during infection (379). This SpvC-dependent dephosphorylation suppresses the inflammatory response in mice and enhances systemic dissemination of *Salmonella* (380,381). Similarly, secretion of the third *spv* effector SpvD suppresses inflammation during infection through interfering with the nuclear transport of RelA, required for NF-κB signaling (382). Together, the *spv* effectors SpvB, SpvC, and SpvD cooperate to enhance invasive infection through the suppression of host immune responses.

Aside from the *spv* operon, there are several other virulence factors conserved between serovar-specific plasmids. Both the plasmid encoded fimbriae (*pef*) operon and the resistance to complement killing (*rck*) gene are present in multiple serovar-specific plasmids, where they contribute to *Salmonella* adherence during infection (370,383). The *pef* operon mediates *Salmonella* adhesion in mice but is ineffective in adherence for some human epithelial cell lines (384,385). Construction of the plasmid-encoded fimbria is

dependent on a second virulence plasmid gene, suppression of cell division inhibition A (SdiA) regulated gene A (*srgA*), which encodes a disulfide oxidoreductase (386). SrgA specifically assists in protein folding of the fimbria and is also implicated in facilitating disulfide bond formation required for T3SS-2 function (387). The virulence plasmid *rck* gene encodes an outer membrane protein that binds to the epidermal growth factor receptor (EGFR) on epithelial cells to facilitate adherence and invasion in a T3SS-1 independent manner (388,389). Rck also binds to the complement components complement component 9 (C9) and C4b-binding protein, which impairs activation of the host complement pathway (390,391). Similarly, the virulence plasmid encoded reduced serum killing (*rsk)* gene mediates resistance to host complement killing, but by an unknown mechanism(392).

1.3.3. Bacteriophage Encoded Virulence Factors

In addition to pathogenicity islands and plasmids, bacteriophage-associated horizontal gene transfer has substantially contributed to the evolution of *Salmonella* pathogenicity. Several *Salmonella* virulence genes are found in the genetic material associated with bacteriophage insertion, commonly referred to as prophage (393). These prophages differ in size, content, and activity, as some prophage are still active, while others are merely phage remnants. Prophage distribution varies across the *S. enterica* subsp. *enterica* serovars because of this mobile phage activity (394,395). Research investigating the contributions of prophage to *Salmonella* pathogenesis has focused primarily on *Salmonella* ser. Typhimurium, as it is home to several bacteriophage insertion sites (396). Deletion of individual prophage in the Typhimurium serovar, specifically *Gifsy-1* and *Gifsy-2,* is associated with attenuation of *Salmonella* in mice

(397). Many studies have investigated the genes within these prophages and characterized their impact on *Salmonella* virulence. Found below is a brief description of the virulence genes encoded in the main prophages of *Salmonella*.

The prophage *Gifsy-1* spans 50 kb and encodes the virulence factors GogA, GogB, and GipA (398,399). *Gifsy-1* gene A (*gogA*) encodes a redundant protease, similar to *pipA* found in SPI-5, that is secreted via the T3SS-2. As with PipA, GogA cleaves RelA and RelB to prevent activation of NF-κB signaling and impairs inflammation during infection (362). GogB is also implicated in suppressing NF-κB activation. Following secretion via the T3SS-2, GogB interacts with S-phase kinase-associated protein 1 (SKIP1) and the human F-box only 22 (FBX022) protein to interfere with NF-κB inhibitor-alpha (IκBα) ubiquitination and prevent activation of the inflammatory response (400). This GogB-dependent suppression of inflammation is linked to greater systemic dissemination of *Salmonella* ser. Typhimurium in mice. The final *Gifsy-1* virulence factor growth in Peyer's patches A (GipA) enhances *Salmonella* survival in the Peyer's patches of orally inoculated mice, although its molecular contributions remain undefined (401).

The prophage *Gifsy-2* similarly spans 50 kb and encodes the virulence factors GtgA, GtgE, SodCI, and SseI (398,399). *Gifsy-2* gene A (GtgA) is a functionally homologous protease to both to the T3SS-2 effectors PipA and GogA as it suppresses NF-κB via cleavage of RelA and RelB (362). The *Gifsy-2* gene *gtgE* encodes a protease that cleaves the human small GTPases Rab29, Rab32, and Rab38 (402,403). During infection, GtgE is secreted by the T3SS-2 and specifically targets Rab-GTPases bound to GDP for cleavage (404). It is proposed that the T3SS-2 effector SopD2 works

cooperatively with GtgE to inactivate Rab-GTPases via its GAP activity. The third *Gifsy-2* virulence factor, SodCI, is a superoxide dismutase (SOD) that is upregulated during infections in macrophages to protect against oxidative bursts in the phagosome (405,406). Deletion of *sodCI*, along with its chromosomal equivalent *sodCII*, attenuates *Salmonella* in mice (407). The final *Gifsy-2* virulence factor, SseI is a deaminase secreted by the T3SS-2 that modulates dendritic cell migration (408). Following secretion, SseI deamidates heterotrimeric G_i proteins increasing host cell survival in macrophages and chemotaxis inhibition in dendritic cells (409). This SseI-dependent modulation of dendritic cell migration is an important regulator of progression to invasive infection.

The prophage *Gifsy-3* was identified shortly after the discovery of *Gifsy-1* and *Gifsy-2*. *Gifsy-3* is present in *Salmonella* ser. Typhimurium strain 14028 and encodes the secretion effectors *Salmonella* anti-inflammatory response activator (SarA) and SspH1 (399). SarA is secreted by both the T3SS-1 and the T3SS-2 and is responsible for activating the STAT3 signaling pathway to induce expression of the anti-inflammatory cytokine IL-10 (410). Following secretion, SarA mimics the cytokine co-receptor gp140 and binds to STAT3 with higher affinity, facilitating its phosphorylation and activation (411). The second *Gifsy-3* effector, SspH1, is an E3 ubiquitin ligase that is secreted by both T3SS-1 and T3SS-2 (412). SspH1 possesses substantial homologous to SspH2, which is encoded in a phage remnant within SPI-12 (344). While the role of either E3 ubiquitin ligase during infection is ambiguous, deletion of both SspH1 and SspH2 attenuates *Salmonella* in a calf infection model (412).

Several other individual virulence factors are found in prophage elements within the *Salmonella* genome. The prophage SopEΦ, which is variably distributed among

Salmonella ser. Typhimurium isolates, encodes the well characterized T3SS-1 effector SopE (413). The functionally homologous T3SS-1 effector SopE2 is also found in a prophage, although the phage is no longer active (205). The T3SS-2 effector SseK3, an arginine glycosyltransferase, is encoded in the prophage ST64B (414). SseK3, along with its chromosomal relative SseK1, glycosylates different components of the death receptor signaling pathway to suppress necroptotic cell death in macrophages (415,416). Antigenic variation is also modulated by prophage elements as the gene clusters *gtr* and *rfb* from the phage ε34 and P22, respectively, encode glycosyltransferases that target the O-antigen (417). These prophage gene clusters are a driving force for LPS diversity in *Salmonella* (418,419).

1.4. The Ubiquitin System

Post-translational modifications (PTMs) govern protein function in a multitude of ways, including stability, activity, and localization. Both prokaryotes and eukaryotes regulate protein function similarly through PTMs involving the attachment of small molecules (ex. phosphorylation, acetylation, and methylation). However, prokaryotes and eukaryotes differ in their PTMs involving the attachment of peptides and small proteins. In particular, the 8.5 kilodalton (kDa) protein ubiquitin is a PTM exclusive to eukaryotes (420), whereas prokaryotes employ a variety of ubiquitin-like modifiers such as prokaryotic ubiquitin-like protein (PUP) (421), Ubiquitin bacterial (Ubact) (422), and small archaeal modifier protein (SAMP) (423). In eukaryotes, ubiquitination is traditionally linked to protein turnover as ubiquitin often acts as a signal for proteasomal degradation (424). While true, ubiquitination regulates several protein functions through ubiquitin linkage variation. Ubiquitination can involve the attachment of a single

ubiquitin to a single residue (monoubiquitination), a single ubiquitin to multiple residues (multi-monoubiquitination), or multiple ubiquitin proteins to a single residue in a linear or branched confirmation (425). These variations in ubiquitination form a complex regulatory system to modulate protein function. The type of ubiquitination performed on a protein is dictated by the enzymes that facilitate the process.

1.4.1. Process of Ubiquitination

Ubiquitination is facilitated by three enzyme types: ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2), and ubiquitin ligases (E3). E1 enzymes are responsible for the first step of ubiquitination, known as ubiquitin activation. Ubiquitin activation is a nonspecific process as the human genome only encodes two ubiquitin specific E1 enzymes, ubiquitin-like modifier-activating enzyme 1 (UBA1) and UBA6 (426). Each E1 enzyme contains three domains important to activating ubiquitin: an adenylation domain, a catalytic cysteine domain, and a ubiquitin fold domain (427). Ubiquitination begins with an E1 enzyme binding free ubiquitin and facilitates adenylation at its carboxyl-terminus (C-terminus) in an adenosine triphosphate (ATP)-dependent manner. The E1 catalytic cysteine then attacks the adenylated ubiquitin to form a thioester bond between the enzyme and the ubiquitin. While the catalytic cysteine is covalently linked to activated ubiquitin, the E1 enzyme can adenylate an additional ubiquitin to restart the process (428). The process continues as the E1 enzyme interacts with an E2 enzyme via its ubiquitin fold domain to facilitate the transfer of the covalently bound activated ubiquitin.

E2 enzymes facilitate ubiquitin conjugation and function as an intermediate between activation and ligation. Relative to ubiquitin activation, ubiquitin conjugation is

a more specific process as the human genome encodes approximately 40 E2 enzymes (429). Upon interacting with an E1-ubiquitin conjugate, the E2 enzyme facilitates transfer of the activated ubiquitin to its own catalytic cysteine via a transthiolation reaction. The newly formed E2-ubiquitin conjugate will then interact with one of many E3 ubiquitin ligases to facilitate the transfer of ubiquitin to a targeted protein (430). Ligation attaches ubiquitin to the target protein, usually on a lysine residue, via the formation of an amide isopeptide bond. This bond is formed between the C-terminal glycine of the ubiquitin and the lysine amide group of the substrate (431). Ubiquitin ligation confers further target specificity as there are over 600 E3 ubiquitin ligases encoded in the human genome (432). These ubiquitin ligases are separated into three main grouping based on their mechanistic differences in ubiquitination: HECT, RING, and RBR. These three types of E3 ubiquitin ligases will be described in further detail in the following section.

1.4.2. Eukaryotic E3 Ubiquitin Ligases

A majority of the E3 ligases encoded in the human genome are from the really interesting new gene (RING) family. All RING-type E3 ligases facilitate ubiquitination by functioning as a scaffold for E2-ubiquitin conjugates and protein substrate binding (Figure 1.4A). As a scaffold, RING-type E3 ligases mediate isopeptide bond formation directly from the ubiquitin charged E2 to the protein substrates (433). Although the RING family transfers ubiquitin in a similar manner, there are several mechanistic differences that separate the E3 ligases. Based on variations in E2-ubiquitin binding, the RING family is divided into two groups, RING and UFD2 homology domain (U-box) E3 ligases. In RING E3 ligases, a series of cysteine and histidine residues form a cross-brace
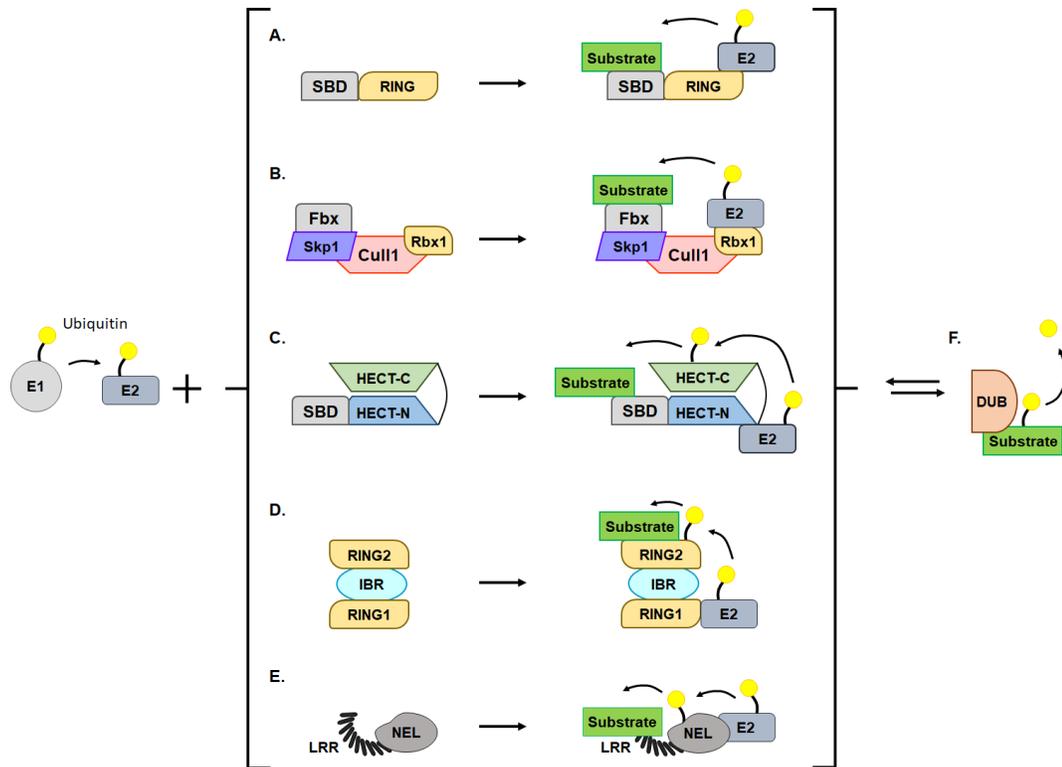
Figure 1.4: E3 ubiquitin ligase classes
Ubiquitination begins with ubiquitin (Ub) activation facilitated by the E1 enzyme followed by ubiquitin conjugation regulated by the E2 enzyme. Ubiquitin ligation is governed by several different E3 ubiquitin ligase classes that are depicted here. (A) RING E3 ligases contain a catalytic RING domain and a substrate binding domain (SBD). The RING domain functions as a scaffold for E2-Ub binding and transfers the Ub directly to the substrate. (B) Cullin-RING E3 ligases are composed of a cullin scaffold protein that binds interchangeable components, as evident with the Skp1-Cull1-F-box protein (SCF) family. Cull1 binds a small RING protein (Rbx1), which binds the E2-Ub conjugate and facilitates ligation to the substrate attached to the F-box protein. (C) HECT E3 ligases contain an SBD and a HECT domain separated into two segments, E2 binding HECT-N and catalytic HECT-C. Following E2-Ub conjugate binding at HECT-N, a stable Ub intermediate is generated at HECT-C before completing the ligation to the substrate. (D) RING-in-between-RING (RBR) E3 ligases contain an in-between RING (IBR) domain flanked by two RING domains (RING1 & 2). Like HECT E3 ligases, RING1 binds the E2-Ub conjugate and transfers the Ub to RING2, generating a stable Ub intermediate. The catalytic RING2 domain then ligates Ub to the substrate. (E) NEL-type E3 ligases possess a leucine rich repeat (LRR) domain and a NEL domain. In the absence of substrate, the LRR domain inhibits NEL activity. Once substrate is bound to the LRR, the catalytic NEL domain transfers Ub from the E2-Ub conjugate to itself, forming a stable Ub intermediate, and then to the substrate. (F) Like other post-translational modification, ubiquitination is a reversible process as deubiquitinases (DUBs) can cleave ubiquitin from substrates via cysteine- or metalloprotease activity. Figure modified form Lin and Machner, 2017 (434).

structure with two zinc ions that functions as a binding platform for an E2-ubiquitin conjugate (435). In U-box E3 ligases, the zinc-binding sites are replaced by hydrogen-bonding and salt bridges to maintain a similar E2-ubiquitin conjugate binding activity (436). Both RING E3 and U-box E3 ligases form homo and heterodimers, with the former increasing ubiquitination efficiency via the binding of an additional E2-ubiquitin conjugate (437). While these two RING family members function as mono or dimeric proteins, another RING type E3 ligase requires multiple components.

The most well characterized multimeric RING E3 family member is the cullin-RING ligase (CRL) class. CRLs are comprised of a cullin protein, a small RING protein, and a substrate adaptor protein (Figure 1.4B). The cullin protein serves as a CRL scaffold that binds a small RING protein at its C-terminus to recruit the E2-ubiquitin conjugate and an adaptor protein at the N-terminal region, which governs substrate specificity (438). The interchangeable nature of adaptor protein binding to cullin proteins confers a wide substrate specificity for CRL-associated ubiquitination. This interchangeable binding is best demonstrated by the SKIP1-Cull1-F-box protein (SCF) family, in which approximately 69 different F-box proteins can be recruited for substrate binding (439). As a result, the SCF family governs several cellular processes including DNA damage repair, genome stability, cell cycle and DNA replication (440). Ubiquitination in these processes is tightly regulated as CRLs require the attachment of the ubiquitin-like modification neural precursor cell expressed developmentally down-regulated protein 8 (NEDD8) for activation of ligase activity (441).

The Homologous to E6AP C-Terminus or HECT-type E3 ligase is the second largest E3 group. Although the HECT family is quite small relative to the RING E3 class,

they are mechanistically distinct. HECT E3 ligases possess a C-terminal HECT domain that is comprised of three important segments: a catalytic C-terminus, a bulky amino-terminus (N-terminus) that governs both E2 and substrate binding, and a flexible hinge that connects both segments (442). Unlike the RING E3 family, HECT E3 ligases facilitate ubiquitination in a two-step process using a catalytic cysteine in the C-terminus (Figure 1.4C). Following the recruitment and binding of an E2-ubiquitin conjugate to the N-terminus of the HECT domain, the C-terminus will perform a transthiolation reaction to transfer to the ubiquitin to its own catalytic cysteine. The C-terminus will then facilitate isopeptide bond formation with the substrate attached to the N-terminus of the HECT domain (443). Aside from the HECT domain, additional domains are also located at the N-terminus of the E3 ligase and are used to classify HECT E3 family into three subfamilies: NEDD4, HERC, and "other". NEDD4 (Neural precursor cell-expressed developmentally downregulated gene 4) family members encode an N-terminal membrane-binding C2 domain and two to four substrate recognition WW domains, in addition to HECT domain (444). The HERC family encodes GEF activity in regulators of chromatin condensation 1 (RCC)-like domains (RLD) which contributes to membrane trafficking (445). The last sub-family "other" shares no specific domains at the N-terminus of the enzyme (446).

The final major E3 ubiquitin ligase class is the RING-in-between-RING (RBR) family. Despite a resemblance in name to the RING family, members of the RBR family perform ubiquitination in a two-step process (Figure 1.4D). Like the HECT domain, RBR E3 ligases are comprised of three conserved components: an E2 binding RING1 domain, an in-between RING (IBR) domain, and a catalytic RING2 domain (447). RBR

associated ligation begins with an E2-ubiquitin conjugate binding to the RING1 domain followed by ubiquitin transfer to the RING2 catalytic cysteine (448). Ubiquitin is then transferred to the protein substrate by an unresolved mechanism, as the substrate binding location in RBR E3 ligases is unknown. Research on the RBR E3 ligases Parkin and the linear ubiquitin chain assembly complex (LUBAC) has furthered our understanding of RBR function, including auto-inhibition of RBR catalytic activity (449,450). However, a complete mechanistic explanation of RBR-associated ubiquitination remains elusive.

1.4.3. Deubiquitinases

Like many PTMs, ubiquitination is a reversible process. While kinases are antagonized by phosphatases for phosphorylation, ubiquitin ligases are countered by deubiquitinases, or DUBs, for ubiquitination. DUBs regulate ubiquitin activity both from a modification standpoint, as well as in *de novo* synthesis. When ubiquitin is expressed, it is translated as a precursor and is processed by DUBs to yield active ubiquitin (451). When ubiquitin is transferred to a protein, DUBs can remove or alter the modification to modulate protein activity and to recycle ubiquitin (Figure 1.4F). This ubiquitin processing is linked to DUB protease activity, which divides the enzyme into two classes: cysteine proteases and metalloproteases (452). The human genome encodes approximately 100 DUBs, a majority of which are cysteine proteases (453). DUBs are additionally classified by their substrate specificity as some DUBs are protein target specific, while others are ubiquitin chain type restricted. This substrate specificity is governed by many factors including protein-binding domains, deubiquitinating complexes, and additional PTMs (454–456). Altogether, the elaborate regulation of

ubiquitination from activation and ligation to deubiquitination reflects the complexity of cellular processes involving ubiquitin.

1.4.4. Function of Ubiquitination

Protein ubiquitination represents a multifaceted regulatory signal that controls the stability, localization, and activation of numerous cellular proteins. The signals provided by ubiquitination can vary based on the site of ubiquitination, the number of modifications, and the chain type (Figure 1.5). In its simplest form, ubiquitination of a single residue, or monoubiquitination, can modulate a range of processes, including DNA repair (457), receptor endocytosis (458), protein interactions (459), transcription (460), and degradation (461). Monoubiquitination at multiple residues, or multi-monoubiquitination, is often associated with cell receptor endocytosis, as demonstrated with ubiquitinated receptor tyrosine kinases (RTKs) following their activation(462). Aside from endocytosis, there is evidence suggesting that multi-monoubiquitination regulates enzymatic activity and protein degradation (463,464).

The addition of multiple ubiquitin modifications in succession to a single substrate residue generates a polyubiquitin chain. The composition of these chains varies based on the lysine residue used to connect each ubiquitin, as there are seven internal lysine residues in ubiquitin (K6, K11, K27, K29, K33, K48, and K63) that serve as a potential ubiquitination sites (424). Polyubiquitin chains that link each ubiquitin via the same lysine residue are referred to as homotypic chains (425). These chains are linked to distinct cellular processes. For example, K48 homotypic chains, the first polyubiquitin chain discovered, label protein substrates for degradation via the ubiquitin proteasome

A. Monoubiquitination / Mult-monoubiquitination / Homotypic Polyubiquitination / Heterotypic Polyubiquitination

B. M_1QIFVK_6TLTGK_{11}TITLEVEPSDTIENVK_{27}AK_{29}IQDK_{33}EGIPPDQQRLIFAGK_{48}QLEDGRTLSDYNIQK_{63}ESTLHLVLRLRGG

C.

Met1 — NF-κB

Lys6 — Degradation / DNA damage / Mitophagy

Lys11 — Degradation / Cell cycle / TNFα

Lys27 — Degradation / DNA damage

Lys29 — Degradation / Wnt/β-Catenin

Lys33 — Degradation / DNA Damage / Golgi trafficking

Lys48 — Degradation

Lys63 — NF-κB / DNA damage

Lys63/Met1 — NF-κB

Lys11/Lys48 — Degradation

Figure 1.5: Ubiquitination chain type and their associated functions.
(A) Ubiquitin is attached to substrates as a monomer, at a single lysine or multiple lysines, or as a polyubiquitin chain. Polyubiquitination can generate homotypic chains (every ubiquitin attached by the same residue) or heterotypic chains (mixture of different attachments). The mixture of different ubiquitin attachments can generate branched ubiquitin chains. Each type of ubiquitination directs a variety of cellular fates. (B) Polyubiquitin chains originate through attachments to one of seven internal ubiquitin lysine residues or the first methionine residue. (C) There are eight different homotypic ubiquitin chain types each with their own associated functions. All homotypic chains, save for M1 and K63, contribute to proteasomal degradation. The most common heterotypic chains K63/M1 and K11/K48 (in the red box) regulate NF-κB signaling and proteasomal degradation, respectively.

system (UPS) (465,466). Specifically, K48-linked substrates bind to Rpn1, the primary receptor of the UPS, where the ubiquitin chain is recycled in a DUB-dependent manner and the protein is subjected to proteolysis within the proteasome core (467). Ubiquitination is a critical regulator of protein turnover as K48 chains account for a majority of ubiquitin linkages in cells (468). Furthermore, most other ubiquitin chains also contribute to proteasomal degradation (469). However, these non-K48 chains modulate several other cellular processes.

K63 homotypic chains are regarded as regulatory ubiquitin linkages because they are not associated with degradation (469). Instead, K63-chains play an integral role in both NF-κB signaling and DNA damage repair. In NF-κB signaling, the RING E3 ligase tumor necrosis factor receptor (TNF) associated factor 6 (TRAF6) generates a K63-chain on the IκB kinase complex (470). The K63 chain recruits tumor growth factor (TGF)-β-activated kinase 1 MAPK kinase 7-binding protein 2/3 (TAB2/3) and NF-κB essential modulator (NEMO), which activate the IκB kinase (IKK) complex leading to downstream phosphorylation IκBα required for NF-κB signaling (471,472). In response to DNA damage, K63 chains are generated at affected sites leading to the recruitment of DNA repair enzymes (473). Several other chain types are also implicated in the DNA damage response including K6, K27, and K33(474,475). K6 and K33 linkages have additional roles in mitophagy (476) and trans-Golgi network trafficking (477), respectively. K11 linkages are also involved in several cellular processes including TNF-α signaling (478) and cell cycle regulation (479). The final lysine chain type, K29, is primarily associated with proteasomal degradation (469,480), but is involved in interfering with the Wnt/β-Catenin signaling pathway (481). Polyubiquitin chains also

assemble independently of internal lysine residues as demonstrated by M1-linear chains that use the N-terminal methionine of ubiquitin for linking (482). The RBR E3 ligase LUBAC is responsible for generating these chains, which facilitate activation of the NF-κB signaling pathway (483).

There are still many uncertainties regarding the impact of ubiquitination on protein function. For instance, polyubiquitin chains involving a mixture of different lysine/methionine linkages, known as heterotypic chains, immensely increase the number of possible chains that uniquely influence protein activity (424). This number is increased even further with the addition of branched ubiquitin chains, where multiple linkages stem from a single ubiquitin modification (484). Some heterotypic chains have a clear impact on protein function such as K48/K11 chains which enhance substrate degradation (485) and K63/M1 chains which facilitate NF-κB signaling (486). Still, an overwhelming majority of heterotypic chains remain without an ascribed function. Another uncertainty regarding ubiquitination involves how polyubiquitin chains are formed. Evidence suggests that E3 ligases dictate chain type, as demonstrated by the C-terminus of HECT E3 ligases which is solely responsible for linkage specificity (487). However, multiple E2 conjugating enzymes are also implicated in the assembly of specific ubiquitin chains (488–490). Therefore, it is more likely that ubiquitin chain type is dictated by a variety of elements on a contextual basis, rather than one factor alone.

1.4.5. Bacterial Subversion of the Ubiquitin System

In the context of bacterial infection, ubiquitination plays an important role in immune signaling pathways. It no surprise that bacteria have evolved strategies to interrupt these signaling pathways for their own benefit. Bacterial inhibition of ubiquitin-

dependent signaling pathways is typically associated with secreted protein effectors. In *Shigella flexneri*, several T3SS effectors are associated with suppressing innate immunity through interfering with host ubiquitination processes. For example, the T3SS effector outer *Shigella* protein I (OspI) deamidates the host E2 enzyme ubiquitin conjugating enzyme 13 (Ubc13), impairing its interaction with the E3 ligase TRAF6 and subsequently suppresses NF-κB signaling (491,492). *S. flexneri* also suppresses NF-κB signaling via secretion of the effector OspZ, a functional homolog of the methyltransferase non-locus of enterocyte effacement encoded effector E (NleE) from enteropathogenic *Escherichia coli* (EPEC) (493). OspZ methylates the K63 binding domain of TAB2/3 preventing activation of the IKK complex (494). *Salmonella* also secretes several effectors that interfere or co-opt host ubiquitin signaling pathways to facilitate pathogenesis. As mentioned earlier in section 1.3, the effectors GogB and SteD both manipulate host E3 ligases to suppress NF-κB signaling (400) and mMHCII antigen presentation (308), respectively. Aside from interfering with host ubiquitination pathways, bacterial pathogens have acquired their own ubiquitin ligases to subvert host signaling processes.

1.4.5.1. Bacterial Encoded E3 Ubiquitin Ligases

Bacterial encoded E3 ubiquitin ligases (BELs) hijack the host ubiquitin pathway to facilitate pathogenesis. While bacteria do not encode ubiquitin, E1 activating enzymes, or E2 conjugating enzymes, BELs have evolved to commandeer host ubiquitination components via mimicry of eukaryotic E3 ligases (495). This subversion of eukaryotic ubiquitination is performed by several bacterial pathogens via the secretion of BELs. As mentioned previously in section 1.3, the *Salmonella* T3SS-1 HECT-like E3 ligase effector SopA ubiquitinates TRIM 56 and TRIM 65, leading to their degradation and

subsequent suppression of IFN-β signaling (233). The HECT-like BEL NleL found in enterohaemorrhagic *E. coli* (EHEC) also interferes with immune signaling. Following its secretion, NleL ubiquitinates JNK, subsequently impairing the transcriptional activity of activator protein-1 (AP-1) and enhancing EHEC pathogenesis (496). Recently, Sheng *et al.* demonstrated that NleL also ubiquitinates several factors required for NF-κB signaling, suggesting that NleL interferes with multiple innate immunity pathways (497).

In addition to HECT E3 mimicry, several pathogens encode BELs that resemble RING family E3 ligases. The most well characterized RING-family BEL is the avirulence protein B from the plant pathogen *Pseudomonas syringae* pv *tomato*, AvrPtoB (498). In *Arabidopsis* infections, AvrPtoB-associated ubiquitination is responsible for the degradation of several kinases and the pattern recognition receptor (PRR) non-expresser of PR gene 1 (NPR1) to subvert plant innate immunity (499–502). RING E3 activity is also observed in a group of 14 homologous BELs in EHEC referred to as the NleG family (503). Two BELs from this family, NleG2-3 and NleG5-1, ubiquitinate proteins involved in cellular metabolism and TGF- β signaling, leading to their degradation (504). While both HECT and RING-type BELs effectively manipulate ubiquitination to enhance pathogenesis, another BEL class has evolved to exploit the host ubiquitin system without mimicry.

1.4.5.2. Novel E3 Ubiquitin Ligases

The novel E3 ubiquitin ligase (NEL) domain is a structurally unique α-helical fold that was first identified in the *S. flexneri* T3SS effector IpaH1.4 (505). Although several bacterial effectors that contain the NEL domain exhibit E3 ligase activity, they bear little structural resemblance to eukaryotic E3 enzymes. NEL family E3 ligases share a

characteristic tertiary structure that is distinct from the HECT and RING E3 families, with a leucine rich repeat (LRR) domain located at the N-terminus followed by the NEL domain at the C-terminus (506,507). The LRR domain is highly variable between NEL family members as it encodes substrate binding specificity (507). In the absence of substrate binding, the LRR domain suppresses ubiquitination activity through an auto-inhibition mechanism (508). This LRR-dependent regulation of catalytic activity is believed to prevent ubiquitination of off-target substrates to enhance the specificity of NEL-family E3 ligases. The catalytic activity of NEL-type BELs is encoded in the highly conserved C-terminal NEL domain (Figure 1.3E). Like HECT E3 ligases, NEL family members use a catalytic cysteine to facilitate ubiquitination in a two-step process (509). Several BELs with NEL-type E3 ligase activity have been identified, with the largest group found in *S. flexneri*. A brief review of NEL family E3 ligases and their impact on bacterial pathogenesis is presented in the following sub sections.

1.4.5.2.1. *Shigella flexneri* and the IpaHs

The genus *Shigella* encodes a group of NEL-type E3 ligases referred to as the invasion plasmid antigen H (IpaH) family. The family includes twelve members that are separated based on their gene location. The first group, *ipaH1.4*, *ipaH2.5*, *ipaH4.5*, *ipaH7.8*, and *ipaH9.8*, are found in the *Shigella* virulence plasmid (510), whereas the second group, *ipaH0722*, *ipaH0877*, *ipaH1383*, *ipaH1880*, *ipaH2022*, *ipaH2202,* and *ipaH2610*, are encoded in the chromosome (511). The chromosomal IpaHs are believed to be redundant as their individual deletion does not impact virulence in mice, while a mutant lacking all five genes is attenuated during infection (511). Alternatively, the importance of the virulence plasmid in *Shigella* pathogenesis has increased focus on the

molecular impact of the plasmid encoded IpaHs during infection (510). Rohde *et al.* first demonstrated the E3 ligase activity of the IpaHs, specifically with IpaH9.8 in yeast as it ubiquitinated the MAPK Ste7, leading to its degradation (512). Several groups have since linked plasmid encoded IpaHs to the ubiquitination of signaling factors involved in human innate immunity. For example, IpaH9.8 and IpaH4.5 were implicated in the ubiquitination of NEMO (513) and RelA (514) from the NF-κB signaling pathway, respectively. However, further studies have failed to replicate these findings (450,515). Three groups have since independently demonstrated that IpaH9.8 is responsible for the ubiquitination and degradation of guanylate-binding proteins (GBPs), impairing IFN-γ stimulated host immunity (516–518). Several other IpaHs are also involved in interfering with host cellular processes including IpaH7.8-dependent degradation of glomulin (GLMN) stimulating inflammasome activation (519,520), and IpaH1.4-dependent degradation of heme-oxidized IRP2 ubiquitin ligase (HOIL) interacting protein (HOIP) from the RBR E3 ligase LUBAC, resulting in impaired NF-κB signaling (450,515), and IpaH4.5-dependent degradation of TRAF family member-associated NF-κB activator-binding kinase 1 (TBK1), inhibiting IFN production (521). Altogether, the IpaH family represents a collection of E3 ligases that subvert a variety of host signaling pathways.

1.4.5.2.2.   *Yersinia* spp., YopM, and YopM-like Effectors

The *Yersinia* outer membrane protein M (YopM) is a virulence plasmid encoded T3SS effector found in *Yersinia* spp. that possesses an LRR domain similar to those found in NEL-type E3 ligases. Unlike like NEL-family BELs, YopM does not possess a C-terminal NEL domain(507,522). Instead of E3 activity, YopM regulates the function of several host factors including serine/threonine-protein kinase N1 (PKN1), PKN2,

ribosomal S6 kinase 1 (RSK1), RSK2 (523,524), capase-1 (525), and IQ motif containing GTPase-activating protein 1 (IQGAP1) (526). These interactions prevent host signaling cascades resulting in the suppression of inflammasome activation in response to *Yersinia* infection (527,528). Recently, Wei *et al.* reported that YopM from *Yersinia pestis* possesses E3 ligase activity, specifically transferring K63 linkages to Nod-like receptor (NLR) family pyrin domain containing 3 (NLRP3) to induce necrosis (529). While YopM lacks a NEL domain, there is heterogeneity in *yopM* sequences between *Yersinia* spp. (530). Additionally, several YopM homologs are encoded in the chromosome of *Yersinia* spp. and contain either a full C-terminal NEL-domain or relic (531). Two of these homologous, *Yersinia* leucine-rich repeat protein A (YlrA) and YlrC, possess functional catalytic cysteines and enhance *Y. pestis* growth in both *in vitro* and *in vivo* infection (532). Therefore, additional research is warranted to investigate the putative E3 ligase activity of YopM and YopM homologs.

1.4.5.2.3. *Salmonella* Secreted NEL-type E3 Ligases

The genus *Salmonella* encodes three BELs from the NEL E3 ligase family, *Salmonella* leucine-rich repeat protein (*slrP*), *sspH1*, and *sspH2* (495). These three E3 ligases are encoded in separate mobile elements in the *Salmonella* chromosome and regulate a variety of cellular processes through their catalytic activity. The E3 ligase SlrP is secreted by both the T3SS-1 and T3SS-2(533), and interacts with both the oxidoreductase thioredoxin (Trx) and the chaperone endoplasmic reticulum DnaJ homologue 3 (ERdj3). SlrP-dependent ubiquitination impairs the reducing activity of Trx during *Salmonella* infection but does not lead to its degradation (534). Conversely, SlrP binding to ERdj3 suppresses the chaperone's capacity to bind denatured substrates in a

ubiquitin independent manner (535). Recently, Rao *el al*. demonstrated that SlrP suppresses IL-1β levels in the small intestine of infected mice leading to a decrease in inflammasome activation (536). Whether SlrP-associated ubiquitination of Trx or binding to ERdj3 contributes to this decrease in IL-1β levels remains to be determined. SlrP is also implicated in regulating host adhesion pathways after proteomic analysis revealed differential expression of several adherence proteins in SlrP-transfected cells (537). While SlrP is involved in regulating several cellular processes, how its E3 ligase activity is involved in this regulation is still unclear.

The remaining NEL family E3 ligases of *Salmonella*, SspH1 and SspH2, share high sequence homology and are both expressed from prophage elements (538). Initially, SspH1 and SspH2 were believed to possess redundant functions as individual gene deletions exhibited no difference in calf infection virulence, whereas a double mutant was significantly attenuated (412). However, further investigations have revealed that SspH1 and SspH2 are quite different. Most *S. enterica* subspecies and serovars encode *sspH2* as it is located within the conserved pathogenicity island SPI-12 (344,539). During infection, SspH2 is secreted by the T3SS-2 and localizes to the cell periphery following its translocation (235). Transfection experiments demonstrate that SspH2 ubiquitinates nucleotide-binding oligomerization domain-containing protein 1 (NOD1), thereby activating the cytosolic receptor and inducing IL-8 expression (540). Recently, Shappo *et al.* showed that SspH2 also downregulates several pro-inflammatory cytokines and increases IL-8 expression during tissue culture and mouse model infections (541). How the E3 activity of SspH2 regulates inflammation during infection remains to be determined.

Unlike *sspH2*, *sspH1* is found in a minority of *Salmonella* subspecies and serovars as the *Gifsy-3* prophage is sparsely distributed (412,539). During infection, SspH1 is secreted by both the T3SS-1 and T3SS-2 and localizes to the nucleus following its translocation (235). Inside the host, SspH1 interacts with the serine/threonine kinase PKN1 (542). Transfection experiments demonstrate that PKN1 is degraded in response to SspH1-dependent ubiquitination resulting in impaired androgen receptor (AR) signaling (543); however, the mechanism of AR signaling suppression and its impact on *Salmonella* infection is currently unknown. Secretion of SspH1 is also implicated in suppressing NF-κB activity (235), although the inhibition is independent of the E3 ligase activity suggesting that PKN1 degradation is not involved (543). In contrast to the other *Salmonella* NEL family BELs, SspH1 possesses a bona fide substrate that multiple groups have confirmed is ubiquitinated by the E3 ligase (505,542–544). However, the roles of SspH1 and PKN1 during *Salmonella* infection remain under characterized and require further investigation. The topic of how SspH1 and PKN1 contribute to *Salmonella* pathogenesis is discussed in further detail in section 5 of this thesis.

1.5. Thesis Rationale and Hypotheses

Salmonella is a major foodborne pathogen in both developing and developed countries. Despite advancements in food safety strategies, *Salmonella* continues to be a large contributor to foodborne related hospitalization and deaths in both Canada and the United States (57,545,546). However, not all *Salmonella* contribute to severe health outcomes as there is a large degree of genetic heterogeneity among the 2600 serovars within the genus (547). While myriad genetic elements contribute to the virulence disparity between serovars, we do not completely understand what factors constitute

serious *Salmonella* disease. Many of the genetic elements that may contribute to *Salmonella* pathogenesis remain uncharacterized. Identifying how these potential virulence factors contribute to *Salmonella* disease is essential to understanding virulence disparity between serovars. The work presented in this thesis examines *Salmonella* from molecular, cellular, and food safety perspectives to improve our understanding of *Salmonella* contamination and virulence.

In section 3, I sought to determine the impact of *Salmonella* contamination on microbial food recalls in Canada. On an annual basis, *Salmonella* is responsible for 88,000 cases of foodborne illness and nearly 1000 hospitalizations (548). Many of the reported cases originate from the improper handling or consumption of raw meat (57). However, a portion of these cases stem from contaminated food products that are traditionally viewed as safe for consumption. When these food products are identified as contaminated, they are recalled from retailers to protect consumers from foodborne illness. Outside of recall alerts and major recall statements, there is minimal reporting on microbial-related food recalls in Canada. I hypothesize that *Salmonella* is a major contributor to microbial-related food recalls in Canada.

In section 4, I sought to develop a medium-throughput protozoan model to screen *Salmonella* isolates for virulence phenotyping. Most infection models used to study *Salmonella* pathogenesis are of mammalian origin. While these models enable evaluation of *Salmonella* virulence, they only simulate a portion of the hosts that *Salmonella* encounter. In the environment and on food, *Salmonella* interact with bacterivorous protozoa that share similar bactericidal pathways as macrophages. As an intracellular pathogen, *Salmonella* encode many virulence factors that facilitate evasion or impairment

of these pathways. Several of these virulence factors that are well known contributors to intracellular survival in humans are also important in resistance to protozoan predation (162,169,181). Therefore, I hypothesize that a protozoan infection model can distinguish between virulent and avirulent *Salmonella* isolates.

In section 5, I sought to examine the impact of SspH1-dependent degradation of PKN1 on *Salmonella* pathogenesis. SspH1 carries potential as a diagnostic marker for virulent *Salmonella* as it is found in a minority of serovars and is associated with virulence in mammals (412,539). Evidence suggests that SspH1 ubiquitinates PKN1 resulting in its degradation; however, PKN1 degradation has only been demonstrated in transfection experiments (543). Aside from AR signaling, PKN1 also regulates a variety of other cellular processes that could impact *Salmonella* pathogenesis. Therefore, I hypothesize that SspH1 degrades PKN1 during infection to enhance *Salmonella* pathogenesis.

Chapter 2: Materials and Methods

2.1. Classification of Microbial-Related Food Recalls

The methods used in this section refer to the work presented in section 3.

As stated by the Government of Canada's Recall Policy (POL-0016), a recall is defined as, "a responsible party's removal from further sale or use, or correction, of a distributed product that presents a risk to the health of consumers or violates the Act or the Regulations" (549). A recall event can include the recall of one or more products, but typically include only one kind of food. The Canadian Food Inspection Agency (CFIA) maintains a record of information related to recalled products in the database Issue Management System (IMS) (550). For my analysis of food recalls in Canada, an access to information request was submitted to the CFIA to obtain all records from the IMS pertaining to food recalls as a result of microbial contamination from January 1st, 2000 through December 31st, 2017. A recalled product was defined as microbially contaminated as per the CFIA standards. The CFIA defines a product as microbially contaminated if the food product is contaminated by a micro-organism, such as bacteria, viruses, or parasites that has the possibility to cause adverse health symptoms (551).

For the purposes of this study, microbial-contaminated food products were categorized in accordance to the classification system used by the CFIA. The CFIA categorizes microbial-contaminated food products into 22 different sub-groups based on the contaminating agent (Table 2.1). A food product can be deemed microbially contaminated and sub-categorized if laboratory analysis of the product identifies the presence of one or more pathogens/micro-organism, or microbial toxins, associated with

Table 2.1: Area of concern categories used by the Canadian Food Inspection Agency to classify microbial-contaminated food products.

Area of concern – sub group	Pathogen, toxin, or condition
Pathogenic	*Bacillus cereus*
	Clostridium botulinum
	Clostridium perfringens
	E. coli O157:H7
	E. coli Non-O157:H7
	Listeria monocytogenes
	Pseudomonas aeruginosa
	Salmonella
	Shigella flexneri
	Staphylococcus aureus
	Vibrio parahaemolyticus
Quality	Aerobic colony count
	Coliforms
	Generic *E. coli*
	Mould
	Yeast
Unspecified	Container integrity
	Other
	Undetermined

foodborne illness. In the absence of laboratory confirmation, a food product can be deemed microbially contaminated if an epidemiological association is established between consumption of a product and cases of human foodborne illness. Additionally, food products are deemed microbially contaminated if they are produced, packaged, transported or stored under conditions that could promote the growth of microbes or production of toxins associated with foodborne illness (550). Recalls of foods contaminated with yeast, mould, coliforms, generic *E. coli*, and high aerobic colony counts were included as the CFIA classifies these food products as microbially contaminated. Food products carrying marine biotoxin under microbial contamination

were also included, despite the CFIA not classifying this contamination under microbiological, as the toxins are microbial in origin.

For each recall of microbially contaminated food products, the Office of Food Safety and Recall assigns a recall classification number (I, II, or III) to indicate the relative degree of health risk associated with consuming the contaminated food (550). A Class I recall specifies a situation in which there is a reasonable probability that the use of, or exposure to, the recalled product will cause serious adverse health consequences or death. A Class II recall specifies a situation in which the use of, or exposure to, the recalled product may cause temporary adverse health consequences or where the probability of serious adverse health consequences is remote. A Class III recall specifies a situation in which the use of, or exposure to, the recalled product is not likely to cause any adverse health consequences (552). The health risk assessment posed by each food product recall is conducted by the Food Directorate of Health Canada (553). Factors taken into consideration during the health risk assessment of microbially contaminated food products include the microbial agent present in the product, the potential severity and duration of adverse health effects associated with the microbial agent, the scientific evidence supporting that the food product is contaminated, the level of contamination, the likelihood of illness as a result of consumption of the product, distribution, consumer habits, and populations likely to be exposed to the product (554).

For each recall of microbially contaminated food products in Canada from 2000 to 2017, recall records were reviewed to gather the following information: pathogen or toxin responsible for contamination, recall classification (I, II, or III), year, and product type. Recalls were characterized based on the pathogen or toxin responsible for the

contamination, the year the recall occurred, and the product type recalled. With regards to product type, the CFIA categorizes food recalls by industry codes. Therefore, similar categories were implemented to those used by Dey *et al.* (555) in their study of foods recalled in the United States due to microbiological contamination for years 2003-2011; with the exception of four new categories: "alcoholic beverages", "animal food", "dietary conventional foods and meal replacements", and "vegetables, vegetable products (sprouts)".

2.2. Bacterial Strains and Culture Conditions

Bacterial strains used in research from section 4 and 5 are described in Table 2.2. *Salmonella* and *E. coli* strains were maintained in or on Lysogeny Broth (LB)-Miller (10g/L tryptone, 10g/L NaCl, 5g/L yeast extract) with or without 20g/L agar. LB-Miller media was supplemented with 100 µg/mL carbenicillin, 25 µg/mL kanamycin, or 15 ug/mL gentamicin when necessary. All antibiotics were obtained from BioShop.

For infections, overnight LB-Miller cultures of *Salmonella* were subcultured 1/30 into 3 mL of LB-Miller with a higher NaCl concentration (300 mM) and incubated at 37°C with agitation at 200 revolutions per minute (RPM) until late-exponential phase to induce SPI-1 expression (556,557). *Salmonella* strains were then washed twice with phosphate-buffered saline (PBS, BioShop) and diluted to the required MOI. These growth conditions were used for used for work presented in section 4 and 5.

2.3. Protozoan Cell Lines, Maintenance, and Infections

The methods used in this section refer to the work presented in section 4.

Table 2.2: Bacterial strain list

Bacterial Strain	Relevant properties	Source
DH5α	*Escherichia coli* strain used for complementation cloning	Invitrogen
Stbl3	*E. coli* strain used for short hairpin RNA cloning	Invitrogen
S17-1λpir pRR008	*E. coli* conjugation strain harbouring pBBRIMCS-2 with sacB and λ-Red system	Julie Ryu[1]
S17-1λpir pRR009	*E. coli* conjugation strain harbouring pBBRIMCS-2 with sacB and flippase recombinase enzyme (FLP)	Julie Ryu
SL1344	*Salmonella enterica* subsp. *enterica* ser. Typhimurium laboratory strain	Dr. Levesque[2]
Δ*invA*Δ*sseB*	SL1344	Dr. Levesque
14028	*Salmonella* ser. Typhimurium isolate ATCC 14028	ATCC
Δs*spH1*	*Salmonella* ser. Typhimurium 14028 with a gene deletion in *sspH1*	This research
Δ*sspH1* pWSK129	*Salmonella* ser. Typhimurium Δs*spH1* harbouring the low copy cloning vector pWSK129 (kanamycin resistance)	This research
Δ*sspH1* p*sspH1*	*Salmonella* ser. Typhimurium Δs*spH1* harbouring the pWSK129 vector containing *sspH1*-FLAG	This research
S1	*S. enterica* subsp. *enterica* ser. Hartford Syst-OMICS isolate	Dr. Levesque
S2	*S. enterica* subsp. *enterica* ser. Newport Syst-OMICS isolate	Dr. Levesque
S3	*S. enterica* subsp. *enterica* ser. Enteritidis Syst-OMICS isolate	Dr. Levesque
S5	*S. enterica* subsp. *enterica* ser. Enteritidis Syst-OMICS isolate	Dr. Levesque
S25	*S. enterica* subsp. *enterica* ser. Amager Syst-OMICS isolate	Dr. Levesque
S26	*S. enterica* subsp. *enterica* ser. Ball Syst-OMICS isolate	Dr. Levesque
S27	*S. enterica* subsp. *enterica* ser. Banana Syst-OMICS isolate	Dr. Levesque
S28	*S. enterica* subsp. *enterica* ser. Benger Syst-OMICS isolate	Dr. Levesque

Bacterial Strain	Relevant properties	Source
S29	*S. enterica* subsp. *enterica* ser. Broughton Syst-OMICS isolate	Dr. Levesque
S30	*S. enterica* subsp. *enterica* ser. Canada Syst-OMICS isolate	Dr. Levesque
S31	*S. enterica* subsp. *enterica* ser. Casablanca Syst-OMICS isolate	Dr. Levesque
S32	*S. enterica* subsp. *enterica* ser. Chingola Syst-OMICS isolate	Dr. Levesque
S33	*S. enterica* subsp. *enterica* ser. Cremieu Syst-OMICS isolate	Dr. Levesque
S34	*S. enterica* subsp. *enterica* ser. Daytona Syst-OMICS isolate	Dr. Levesque
S35	*S. enterica* subsp. *enterica* ser. Duesseldorf Syst-OMICS isolate	Dr. Levesque
S36	*S. enterica* subsp. *enterica* ser. Elisabethville Syst-OMICS isolate	Dr. Levesque
S37	*S. enterica* subsp. *enterica* ser. Falkensee Syst-OMICS isolate	Dr. Levesque
S38	*S. enterica* subsp. *enterica* ser. Fresno Syst-OMICS isolate	Dr. Levesque
S39	*S. enterica* subsp. *enterica* ser. Godesberg Syst-OMICS isolate	Dr. Levesque
S40	*S. enterica* subsp. *enterica* ser. Hull Syst-OMICS isolate	Dr. Levesque
S41	*S. enterica* subsp. *enterica* ser. Indikan Syst-OMICS isolate	Dr. Levesque
S42	*S. enterica* subsp. *enterica* ser. Kouka Syst-OMICS isolate	Dr. Levesque
S43	*S. enterica* subsp. *enterica* ser. Luciana Syst-OMICS isolate	Dr. Levesque
S44	*S. enterica* subsp. *enterica* ser. Luckenwalde Syst-OMICS isolate	Dr. Levesque
S45	*S. enterica* subsp. *enterica* ser. Orientalis Syst-OMICS isolate	Dr. Levesque

Bacterial Strain	Relevant properties	Source
S46	*S. enterica* subsp. *enterica* ser. Pasing Syst-OMICS isolate	Dr. Levesque
S47	*S. enterica* subsp. *enterica* ser. Solt Syst-OMICS isolate	Dr. Levesque
S48	*S. enterica* subsp. *enterica* ser. Tado Syst-OMICS isolate	Dr. Levesque
S49	*S. enterica* subsp. *enterica* ser. Taiping Syst-OMICS isolate	Dr. Levesque
S50	*S. enterica* subsp. *enterica* ser. Taksony Syst-OMICS isolate	Dr. Levesque
S51	*S. enterica* subsp. *enterica* ser. Tyresoe Syst-OMICS isolate	Dr. Levesque
S52	*S. enterica* subsp. *enterica* ser. Wentworth Syst-OMICS isolate	Dr. Levesque
S53	*S. enterica* subsp. *enterica* ser. Westhampton Syst-OMICS isolate	Dr. Levesque
S54	*S. enterica* subsp. *enterica* ser. Weston Syst-OMICS isolate	Dr. Levesque
S158	*S. enterica* subsp. *enterica* ser. Oranienburg Syst-OMICS isolate	Dr. Levesque
S172	*S. enterica* subsp. *enterica* ser. Arizonae Syst-OMICS isolate	Dr. Levesque
S187	*S. enterica* subsp. *enterica* ser. Enteritidis Syst-OMICS isolate	Dr. Levesque
S189	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S193	*S. enterica* subsp. *enterica* ser. Thompson Syst-OMICS isolate	Dr. Levesque
S194	*S. enterica* subsp. *enterica* ser. Thompson Syst-OMICS isolate	Dr. Levesque
S195	*S. enterica* subsp. *enterica* ser. Newport Syst-OMICS isolate	Dr. Levesque
S200	*S. enterica* subsp. *enterica* ser. Javiana Syst-OMICS isolate	Dr. Levesque

Bacterial Strain	Relevant properties	Source
S203	*S. enterica* subsp. *enterica* ser. Javiana Syst-OMICS isolate	Dr. Levesque
S204	*S. enterica* subsp. *enterica* ser. Saintpaul Syst-OMICS isolate	Dr. Levesque
S205	*S. enterica* subsp. *enterica* ser. Saintpaul Syst-OMICS isolate	Dr. Levesque
S206	*S. enterica* subsp. *enterica* ser. Muenchen Syst-OMICS isolate	Dr. Levesque
S207	*S. enterica* subsp. *enterica* ser. Muenchen Syst-OMICS isolate	Dr. Levesque
S209	*S. enterica* subsp. *enterica* ser. Braenderup Syst-OMICS isolate	Dr. Levesque
S212	*S. enterica* subsp. *enterica* ser. Stanley Syst-OMICS isolate	Dr. Levesque
S213	*S. enterica* subsp. *enterica* ser. Agona Syst-OMICS isolate	Dr. Levesque
S215	*S. enterica* subsp. *enterica* ser. Agona Syst-OMICS isolate	Dr. Levesque
S219	*S. enterica* subsp. *enterica* ser. Hadar Syst-OMICS isolate	Dr. Levesque
S229	*S. enterica* subsp. *enterica* ser. Derby Syst-OMICS isolate	Dr. Levesque
S236	*S. enterica* subsp. *enterica* ser. Mbandaka Syst-OMICS isolate	Dr. Levesque
S238	*S. enterica* subsp. *enterica* ser. Mbanadak Syst-OMICS isolate	Dr. Levesque
S239	*S. enterica* subsp. *enterica* ser. Montevideo Syst-OMICS isolate	Dr. Levesque
S241	*S. enterica* subsp. *enterica* ser. Montevideo Syst-OMICS isolate	Dr. Levesque
S246	*S. enterica* subsp. *enterica* ser. Kentucky Syst-OMICS isolate	Dr. Levesque
S256	*S. enterica* subsp. *enterica* ser. Bovismorbificans Syst-OMICS isolate	Dr. Levesque

Bacterial Strain	Relevant properties	Source
S267	*S. enterica* subsp. *enterica* ser. Kiambu Syst-OMICS isolate	Dr. Levesque
S269	*S. enterica* subsp. *enterica* ser. Senftenberg Syst-OMICS isolate	Dr. Levesque
S270	*S. enterica* subsp. *enterica* ser. Senftenberg Syst-OMICS isolate	Dr. Levesque
S272	*S. enterica* subsp. *enterica* ser. Litchfield Syst-OMICS isolate	Dr. Levesque
S273	*S. enterica* subsp. *enterica* ser. Litchfield Syst-OMICS isolate	Dr. Levesque
S277	*S. enterica* subsp. *enterica* ser. Uganda Syst-OMICS isolate	Dr. Levesque
S306	*S. enterica* subsp. *enterica* ser. Poona Syst-OMICS isolate	Dr. Levesque
S307	*S. enterica* subsp. *enterica* ser. Poona Syst-OMICS isolate	Dr. Levesque
S317	*S. enterica* subsp. *enterica* ser. Ohio Syst-OMICS isolate	Dr. Levesque
S325	*S. enterica* subsp. *enterica* ser. Bredeney Syst-OMICS isolate	Dr. Levesque
S333	*S. enterica* subsp. *enterica* ser. Berta Syst-OMICS isolate	Dr. Levesque
S334	*S. enterica* subsp. *enterica* ser. Kottbus Syst-OMICS isolate	Dr. Levesque
S354	*S. enterica* subsp. *enterica* ser. Gaminara Syst-OMICS isolate	Dr. Levesque
S361	*S. enterica* subsp. *enterica* ser. Adelaide Syst-OMICS isolate	Dr. Levesque
S364	*S. enterica* subsp. *enterica* ser. Cerro Syst-OMICS isolate	Dr. Levesque
S365	*S. enterica* subsp. *enterica* ser. Cerro Syst-OMICS isolate	Dr. Levesque
S428	*S. enterica* subsp. *enterica* ser. Newport Syst-OMICS isolate	Dr. Levesque
S441	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque

Bacterial Strain	Relevant properties	Source
S488	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S490	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S492	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S493	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S494	*S. enterica* Syst-OMICS isolate	Dr. Levesque
S498	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S499	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S504	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S525	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S537	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S551	*S. enterica* Syst-OMICS isolate	Dr. Levesque
S603	*S. enterica* subsp. *enterica* ser. Bareilly Syst-OMICS isolate	Dr. Levesque
S718	*S. enterica* subsp. *enterica* ser. Derby Syst-OMICS isolate	Dr. Levesque
S761	*S. enterica* subsp. *enterica* ser. Stanleyville Syst-OMICS isolate	Dr. Levesque
S774	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S775	*S. enterica* subsp. *enterica* ser. Typhimurium Syst-OMICS isolate	Dr. Levesque
S776	*S. enterica* subsp. *arizonae* Syst-OMICS isolate	Dr. Levesque
S777	*S. enterica* subsp. *arizonae* Syst-OMICS isolate	Dr. Levesque

Bacterial Strain	Relevant properties	Source
S778	*S. enterica* subsp. *arizonae* Syst-OMICS isolate	Dr. Levesque
S779	*S. enterica* subsp. *arizonae* Syst-OMICS isolate	Dr. Levesque
S781	*S. enterica* subsp. *arizonae* Syst-OMICS isolate	Dr. Levesque
S784	*S. enterica* subsp. *arizonae* Syst-OMICS isolate	Dr. Levesque
S785	*S. enterica* subsp. *arizonae* Syst-OMICS isolate	Dr. Levesque
S787	*S. enterica* subsp. *arizonae* Syst-OMICS isolate	Dr. Levesque
S1288	*S. enterica* subsp. *enterica* ser. Javiana Syst-OMICS isolate	Dr. Levesque
S1393	*S. enterica* subsp. *enterica* ser. Tornow Syst-OMICS isolate	Dr. Levesque
S1395	*S. enterica* Syst-OMICS isolate	Dr. Levesque
S1426	*S. enterica* subsp. *enterica* ser. Berta Syst-OMICS isolate	Dr. Levesque
S1603	*S. enterica* subsp. *enterica* ser. Havana Syst-OMICS isolate	Dr. Levesque
S1925	*S. enterica* subsp. *enterica* ser. Muenchen Syst-OMICS isolate	Dr. Levesque

[1]Julie Ryu, Former Rohde Lab Research Technician, Halifax, Nova Scotia
[2]Dr. Roger Levesque, Université Laval, Quebec City, Quebec

2.3.1. Maintenance of *Tetrahymena* spp.

Tetrahymena spp. (*Tetrahymena pyriformis*, *Tetrahymena tropicalis*, *Tetrahymena thermophila*) were gifted by Dr. Rafael Garduno. *Tetrahymena* spp. were maintained in Plate Count Broth (PCB, 5g/L yeast extract, 10g/L tryptone, 2g/L glucose, pH 7) at 25˚C as previously described by Berk *et al.* (159). An active culture of each *Tetrahymena* species was generated every two weeks by subculturing 1/40 into 40 mL of fresh PCB.

2.3.2. Maintenance of *Dictyostelium*

Dictyostelium strain AX4 (DBS0302402) was obtained from Dicty Stock Center and cultured according to standard procedure (558,559). Briefly, AX4 was maintained at 22˚C on a confluent lawn of *E. coli* DH5α grown on Standard Medium (SM) agar (20 g/L agar, 10 g/L peptone, 10 g/L yeast extract, 10 g/L glucose, 1.9 KH_2PO_4, 1 g/L $MgSO_4 \cdot 7H_2O$, 0.6 g/L K_2HPO_4) (560). Fresh *Dictyostelium* SM agar plates were generated on a fortnightly basis.

2.3.3. Maintenance of *Acanthamoeba* spp. and *Vermamoeba vermiformis*

Amoeba strains *Acanthamoeba castellanii*, *A. polyphaga*, *A. rhysodes*, and *Vermamoeba vermiformis* were gifted by Dr. Rafael Garduno. Axenic cultures of *Acanthamoeba* and *Vermamoeba* cell lines were propagated as trophozoites modified peptone-yeast extract-glucose (mPYG) broth (20 g/L peptone, 18 g/L glucose, 1 g/L yeast extract, 960 mg/L $MgSO_4 \cdot 7H_2O$, 880 mg/L sodium citrate, 350 mg/L Na_2HPO_4, 340 mg/L KH_2PO_4, 44 mg/L $CaCl_2$, 20 mg/L $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) (561) in 25 cm^2 tissue culture flasks at 30˚C until near confluence was reached. Tissue culture flasks containing near confluent amoebae were gently shaken, and culture medium containing nonadherent amoebae was removed. Adherent amoebae were removed by agitating the empty flasks and resuspending with fresh mPYG broth. Each amoebae resuspension was then subcultured 1/10 in mPYG into a new tissue culture flask.

2.3.4. *Tetrahymena* Grazing Assays

Tetrahymena spp. were grown in 2/3 strength PCB for two days at 25˚C prior to grazing assays. On the day of grazing assay, ciliates were incrementally transferred from PCB to Tris-buffered Osterhout's solution (420 mg/L NaCl, 121 mg/L Tris, 34 mg/L

MgCl$_2$·6H$_2$O, 16 mg/L MgSO$_4$·7H$_2$O, 9.2 mg/L KCl, 4 mg/L CaCl$_2$, pH 7) (159). Ciliates were pelleted via centrifugation at 500 x g for 10 minutes at 25°C. Half of the supernatant was carefully removed and replaced with the same volume of Osterhout's solution to obtain a 50% mixture of PCB and Osterhout's solution. Ciliates were left incubating in the new mixture for 15 minutes before repeating the process two additional times. *Tetrahymena* concentrations were subsequently quantified through directing counting of Lugol's iodine-fixed samples with a Neubauer chamber (159). Ciliates were resuspended to a final concentration of 10^4 cells/mL and seeded into 12-well plates. Subcultured *Salmonella* at late-exponential phase were washed twice and diluted PBS to obtain a MOI of 1000. Following inoculation, the 12-well plate was incubated as 30°C for 24 hours. Aliquots of co-culture were taken from each well at 1- and 24-hours post-inoculation. Aliquots were used to measure ciliate replication through counting of Lugol's iodine-fixed samples with a Neubauer chamber. Aliquots were centrifuged at 10,000 x g for 1 minute and resuspended in 0.5% Triton-100X to free intracellular bacteria (159). Culture forming unit (CFU) enumeration was then performed by serial dilution of samples and plating on LB agar.

2.3.5. *A. castellanii* and *V. vermiformis* Grazing Assays

Amoebae were seeded into 12-well plates at a concentration of 10^5 cells/mL and incubated for 24 hours at 30°C to permit adherence to the wells. An hour prior to inoculation, amoeba were washed twice with Page's amoeba saline (PAS, 142 mg/L Na$_2$HPO$_4$, 136 mg/L KH$_2$PO$_4$, 120 mg/L NaCl, 4 mg MgSO$_4$·7H$_2$O, 3 mg/L CaCl$_2$) (562) and maintained in the non-nutrient buffer. Subcultured *Salmonella* at late-exponential phase were washed twice and diluted in PBS to obtain a MOI of 10. Following

inoculation, the 12-well plate was incubated at 30˚C for 24 hours. Aliquots of co-cultures were taken from each well at 1- and 24-hours post-inoculation. Aliquots were serial diluted and plated on LB agar for CFU enumeration.

2.3.6. Assessment of *A. castellanii* Viability

A. castellanii was co-cultured with *Salmonella* as described above. At the 1- and 24- hour time points, LIVE/DEAD *Bac*Light (ThermoFisher Scientific) viability dye mix (SYTO 9 and propidium iodide (PI)) was added to each well and co-cultures were incubated at room temperature in the dark for 15 minutes. Fluorescent staining of amoebae was then assessed at 510 nm (green) and 620 nm (red) using fluorescent microscopy (Olympus Life Sciences Model BX53F, X-Cite 120LED). Percent fluorescent cells was determined using cellSens software (Olympus Life Sciences). Pixel thresholds were increased to exclude fluorescence related to bacterial staining. Amoeba displaying green fluorescence were only designated as SYTO 9, whereas those displaying yellow fluorescence (green and red) were labeled as PI. Uninoculated amoeba were included as a negative control.

2.3.7. *Dictyostelium* Grazing Assays

Dictyostelium were grown at 22˚C with agitation at 180 RPM in HL5 axenic medium (14 g/L tryptone, 14 g/L glucose, 7 g/L yeast extract, 1.2 g/L K_2HPO_4, 350 mg/L Na_2HPO_4, pH 6.3) (169). Amoebae were pelleted in early exponential phase (1 x 10^6 cells/mL) through centrifugation at 500 x g for 10 minutes at 4˚C. The supernatant was discarded, and the pellet was resuspended using Soerensen buffer (2 g/L KH_2PO_4, 290 mg/L Na_2PO_4, pH 6.0) (169). Amoebae were pelleted and washed two additional times. *Dictyostelium* concentration was subsequently quantified through trypan blue exclusion

counting with a Neubauer chamber and diluted to a final concentration of 10^4 cells/mL. Subcultured *Salmonella* at late-exponential phase were washed twice and diluted in PBS to obtain a MOI of 100. Amoeba and *Salmonella* were co-cultured in 10 mL of Soerensen buffer and incubated at 22˚C with agitation at 180 RPM for 24 hours. Aliquots of co-culture were taken from each well at 1- and 24-hours post-inoculation. Aliquots were centrifuged at 10,000 x g for 1 minute and resuspended in 0.5% Triton-100X to free intracellular bacteria (169). CFU enumeration was then performed by serial dilution of samples and plating on LB agar.

2.3.8. *Acanthamoeba* Gentamicin Protection Assays

Amoebae were seeded into a 12-well plate at a concentration of 10^5 cells/mL and incubated for 24 hours at 30˚C to permit adherence to wells. The number of amoebae per well was calculated an additional time prior to infection. Subcultured *Salmonella* at late-exponential phase were washed twice and diluted in PBS to obtain a MOI of 100. Following inoculation, the 12-well plate was centrifuged for 5 minutes at 500 x g to promote contact between bacteria and amoebae. The plate was then incubated at 30˚C for 60 minutes. After incubation, the medium was removed, wells were washed with PBS, and fresh mPYG broth supplemented with 50 µg/mL gentamicin sulfate was added to kill extracellular bacteria. Following incubation for 30 minutes at 30˚C, the wells were washed twice with PBS and lysed in 0.5% sodium deoxycholate (176). CFU enumeration was then determined by serially diluting the samples and plating them on LB agar.

2.4. Investigation of SspH1 and PKN1

The methods used in this section refer to the work presented in section 5.

2.4.1. Primers and Polymerized Chain Reaction

All primers used in this research are described in Table 2.3. *Salmonella*-related primers were designed based on the *S. enterica* subsp. *enterica* ser. Typhimurium 14028 sequence, Genbank accession number CP001363.1 (563). Sequences for generic sequencing primers (T3/T7 promoter primers) were obtained from Addgene (564). Primers used for λ-Red recombination were designed based on previous work with *E. coli* (565). Polymerase chain reactions (PCRs) involving cloning and sequencing were performed using New England Biolabs (NEB) Q5 High-Fidelity DNA polymerase with reaction conditions as suggested by the supplier. PCRs related to λ-Red recombination were conducted using NEB Taq DNA polymerase with the specified supplier reaction conditions. Primer sequences used in construction of PKN1 short hairpin RNA (shRNA) were obtained from the Broad Institute (566). All primers were synthesized by Sigma-Aldrich.

2.4.2. λ-Red Recombination

The *Salmonella sspH1* deletion mutant (Δ*sspH1*) was constructed using λ-red recombination as described in Sidik *et al.* (567). Briefly, a kanamycin knockout cassette was generated through PCR using the plasmid pGEM-kanR as template, which contains a kanamycin resistance gene flanked by flippase recognition targets (FRTs) and priming sites (P1 & P2). Purified PCR product was electroporated into wild type *Salmonella* ser. Typhimurium (14028) containing the plasmid pRR008, which encodes the λ-red recombination machinery from λ phage along with a *sacB* negative selection marker. The electroporated *Salmonella* were grown for an hour in liquid medium at 37°C with

Table 2.3: Primer list

Primer	Description	Sequence (5' – 3')
SspH1_LR1R	Reverse primer to amplify *sspH1* kanR knockout cassette	TCAGTTAAGACGCCACCGGGCTGTCAGATAGCTACCCAGCACCTCTGTAGGCTGGAGCTGCTTCG
SspH1_LR1F	Forward primer to amplify *sspH1* kanR knockout cassette	ATCCGCAATACACAACCTTCTGTAAGTATGCAGGCTATTGCTGGTGATTCCGGGGATCCGTCGACC
SspH1_LR2F	Screening λ-red recombinants	TTCCATCAACTTCGCACTAT
P2_REV	Screening λ-red recombinants	TGTAGGCTGGAGCTGCTTCG
sspH1_FLAG_FOR_XbaI	*sspH1* cloning	TATTCTAGATCACTTGTCGTCATCGTCTTTGTAGTCGTTAAGACGCCACCGGGC
sspH1_P_REV_SacI	*sspH1* cloning	TATGAGCTCGCATCCGGGATATCTGGGGTC
T3R	T3 promoter primer	AATTAACCCTCACTAAAGGG
T7F	T7 promoter primer	TAATACGACTCACTATAGGG
U6	Human U6 promoter primer	GACTATCATATGCTTACCGT
sspH1_Seq_Chk_REV	*sspH1* sequencing	GGAGCTTCAGGTGAAAAC
sspH1_Seq_1_FOR	*sspH1* sequencing	GTTTTCACCTGAAGCTCC
sspH1_Seq_1_REV	*sspH1* sequencing	CTGAGAGCAAAAACCTTTG
sspH1_Seq_2_FOR	*sspH1* sequencing	CAAAGGTTTTTGCTCTCAG
sspH1_Seq_2_REV	*sspH1* sequencing	GTCAGGACTACAGAAGCTG
sspH1_Seq_3_FOR	*sspH1* sequencing	CAGCTTCTGTAGTCCTGAC
sspH1_Seq_3_REV	*sspH1* sequencing	GTTTCACAGAAGGCAGCA
sspH1_Seq_4_FOR	*sspH1* sequencing	TGCTGCCTTCTGTGAAAC
sspH1_Seq_4_REV	*sspH1* sequencing	ATGTTTAATATCCGCAATACAC
sspH1_Seq_5_FOR	*sspH1* sequencing	GTGTATTGCGGATATTAAACAT
sspH1_Seq_5_REV	*sspH1* sequencing	GGTTCCATAAAACGATACTG
sspH1_Seq_Chk_FOR	*sspH1* sequencing	CAGTATCGTTTTATGGAACC
pLKO_PKN1_1859_FOR	Broad Institute forward PKN1 shRNA primer	CCGGACCTCTGACCCTCGAAGATTTCTCGAGAAATCTTCGAGGGTCAGAGGTTTTTTG
pLKO_PKN1_1859_REV	Broad Institute reverse PKN1 shRNA primer	AATTCAAAAAACCTCTGACCCTCGAAGATTTCTCGAGAAATCTTCGAGGGTCAGAGGT

agitation at 200 RPM, then plated on kanamycin containing medium for recombinant selection. Recombination was confirmed through PCR amplification using a primer that anneals to P2 and a primer that binds upstream in the flanking *Salmonella* chromosome region. The kanamycin resistance marker was later removed through conjugation of the plasmid pRR009, which encodes the flippase recombinase enzyme that facilitates recombination between FRT sites flanking the cassette (567).

2.4.3. Plasmid Construction

Plasmids used and constructed in research related to section 5 are described in Table 2.4. The *sspH1* complementation plasmid (p*sspH1*) was constructed in the low copy vector pWSK29 (568) provided by Dr. Samantha Gruenheid. The open reading frame of *sspH1* along with 718 bp of sequence upstream of the start codon was amplified in accordance with work by Haraga and Miller (235), with the addition of a C-terminal FLAG tag. The amplified *sspH1*-FLAG PCR product was cloned into pWSK129 at the SacI and XbaI restriction sites to generate p*sspH1*. Ligated p*sspH1* was then transformed into DH5α for propagation. The insert in pWSK129 was sequenced using multiple primers that anneal within *sspH1* (Table 2.4) to verify the absence of mutations. Cloning of PKN1 shRNA into pLKO.1 TRC was performed in accordance with Addgene pLKO.1 cloning protocol (569). The pLKO.PKN1.shRNA.1.1 vector was sequenced using the U6 primer to confirm the presence of the PKN1 shRNA. All sequencing was performed by GENEWIZ.

2.4.4. Mammalian Cell Culture Maintenance and Treatments

All mammalian cell lines were subcultured at 1/10 every 2-3 days and incubated at

Table 2.4: Plasmid list

Plasmid	Relevant properties	Source
pWSK129	Low copy cloning vector with kanamycin resistance.	Dr. Gruenheid[1]
p*sspH1*	*sspH1* along with native promoter and C-terminal FLAG-tag cloned into pWSK129	This research
pRR008	pBBRIMCS-2 with sacB, λ-Red system, and gentamicin resistance	Julie Ryu[2]
pRR009	pBBRIMCS-2 with sacB. flippase recombinase enzyme (FLP), and gentamicin resistance.	Julie Ryu
pGEM-kanR	pGEM-TEasy vector encoding the kanR resistance cassette.	Julie Ryu
pLKO.1 TRC	Replication-incompetent lentiviral cloning vector with puromycin and carbenicillin resistance.	Dr. McCormick[3]
pLKO.PKN1 shRNA.1.1	Replication-incompetent lentiviral vector encoding PKN1 shRNA with puromycin and carbenicillin resistance	This research
pLKO. Nt.shRNA	Replication-incompetent lentiviral vector encoding non-targeting (Nt) shRNA with puromycin and carbenicillin resistance	Dr. McCormick
psPAX	Lentiviral packaging vector with carbenicillin resistance.	Dr. McCormick
pMD2.G	Vesicular stomatitis virus (VSV)-G envelope expressing vector with carbenicillin resistance.	Dr. McCormick

[1]Dr. Samantha Gruenheid, McGill University, Montreal, Quebec
[2]Julie Ryu, Former Rohde Lab Research Technician, Halifax, Nova Scotia
[3]Dr. Craig McCormick, Dalhousie University, Halifax, Nova Scotia

37°C with 5% CO_2. Both HeLa and HEK293T cells, gifts from Dr. Craig McCormick, and HT29 cells, a gift from Dr. Johan Van Limbergen, were cultured as described above in Dulbecco's modified eagle medium (DMEM) with 100mM sodium pyruvate, 25 mM glucose, 10 mM HEPES, 2 mM L-glutamine, and 10% fetal bovine serum (FBS) (gibco). U937s cells, a gift from Dr. Zhenyu Cheng, were cultured in RPMI medium 1640 with 10 mM HEPES, 2 mM L-glutamine, and 10% heat-inactivated FBS (Gibco). Differentiation of U937 cells was performed as previously described (570). Briefly, seeded U937 cells

were incubated in complete RPMI 1640 with 100 ng/mL phorbol 12-myristate-13-acetate (PMA, BioShop) for 48 hours. Spent medium was then replaced with fresh complete RPMI 1640. All active cell lines were tested for mycoplasma contamination using the Venor™GeM Mycoplasma Detection Kit (Sigma) every two months.

Serum starvation experiments involving differentiated U937 cells were initiated through removing medium and replacing with FBS-free RPMI 1640. Cells were incubated in serum-free medium for 3 hours before either harvesting or replenishing the medium with 10% FBS. U937 cells with replenished serum were harvested after incubating for 15 minutes. Proteasome and caspase activation inhibition were performed through removing medium on differentiated U937 cells and replacing with complete RPMI 1640 with either 10 µM MG132 (Sigma), 10 µM IDN-6556 (SelleckChem), or both. Cells were incubated with inhibitors for 0.5, 1.5, 3 hours before harvesting.

2.4.5. Lentiviral Production and Transduction

HEK293T cells at 80% confluency were seeded at 1/10 in complete medium into 10 cm^2 dishes. The next day, medium was removed from cells, monolayers were washed twice with PBS and replenished with 9 mL FBS-free DMEM. Transfections were performed using polyethylenimine (PEI, Sigma) and used the following reaction components: 18 µL PEI in 500 µL serum-free Opti-MEM (Gibco) per transfection, 3.3 µg pLKO.PKN1.shRNA.1.1 or pLKO.Nt.shRNA, 2 µg psPAX (Dr. Craig McCormick), and 1 µg (Dr. Craig McCormick). Cells were transfected for 5 hours, and medium was replaced with complete DMEM. Supernatants were harvested 24 hours post-transfection, passed through a 0.45 µm filter and stored in 1 mL aliquots at -80˚C.

For transduction, U937 cells were seeded from high confluency into 12-well plates at a density between 2-4 x 10^4 cells/mL. The following day, plates were centrifuged at 500 x g for five minutes and the medium was then replaced with complete RPMI 1640 containing 4 µg/mL polybrene (Sigma). Lentiviral aliquots were thawed at 37˚C and were added to cells in a series of dilutions ranging from 1/2 to 1/2048. A single well remained untransduced to serve a negative control for antibiotic selection. Puromycin (Invitrogen) was added to a concentration of 1 µg/mL to each well 24-hours post-transduction. Puromycin-treated cells were selected for two to three days. The transduced well with lentiviral dilution resulting in approximately 30% U937 cell survival was carried forward.

2.4.6. *Salmonella* Infections in Mammalian Cells

U937 cells were seeded at a density of 10^6 cells/mL in 6-well plates and differentiated for 48 hours prior to infection. Two hours prior to infection, U937 cells were washed twice with PBS and maintained in fresh complete RPMI 1640. One well was trypsinized and the cell number per well was determined through trypan blue exclusion counting using a hemocytometer. Subcultured *Salmonella* at late-exponential phase were washed twice and diluted in PBS to a MOI of 10 or 30. Following inoculation, infected U937 cells were incubated for 30 minutes at 37˚C with 5% CO_2. After 30 minutes, medium was replaced with complete RPMI 1640 containing 50 µg/mL gentamicin and cells were incubated for an additional hour. Following incubation, the medium was replaced one final time with complete RPMI 1640 containing 15 µg/mL gentamicin and cells were incubated until the specified time point for harvesting. Infected U937 cells were harvested at 0.5-, 1-, 1.5-, 2-, 3-, 4-, 8-, and 16-hours post-inoculation

depending on the experiment. Infections requiring caspase activation inhibition involved pre-treatment of differentiated U937 cells with 10 μM IDN-6556 or 10 μM dimethyl sulfoxide (DMSO, Fisher) for 3 hours prior to inoculation and addition of each treatment with every medium replacement during infection.

For quantification of *Salmonella* internalization and intracellular replication, HeLa, HT29, and U937 cells were seeded at a density of 10^6 cells/mL in 6-well plates 48 hour prior to infection, with specific 100 ng/mL PMA treatment for U937 cell differentiation. Infections were performed is the same manner described above. Following the 1-hour incubation with 50 μg/mL gentamicin, cells were either processed for internalization CFU enumeration or maintained in fresh complete medium containing 15 μg/mL gentamicin and incubated until the specified time point. Cells were additionally processed at 4- and 16-hour post initial gentamicin incubation to enumerate CFUs corresponding to intracellular replication. For cell processing, medium was removed and cells were washed once with PBS then lysed with 250 μL/well NP-40 lysis buffer (8.8 g/L NaCl, 6 g/L Tris, 1% NP-40, Cold Spring Harbor). Lysates were then serially diluted and plated on LB agar to enumerate CFUs.

2.4.7. Immunoblotting

Cell lysates were harvested using radioimmunoprecipitation assay (RIPA) buffer (8.8 g/L NaCl, 6 g/L Tris, 184 mg/L sodium orthovanadate, 42 mg NaF mg/L, 1% Triton-X100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, SDS). All cell lines were harvested by removing culture medium, carefully washing wells with ice-cold PBS, adding 200 μL of RIPA buffer to each well, dislodging adherent cells with a cell scrapper, and transferring suspensions to precooled microcentrifuge tubes. Cell

suspensions were agitated at 4˚C for 30 minutes and subsequently pelleted through centrifugation at 13,000 x g for 20 minutes at 4˚C. The supernatant from each cell suspension was transferred to a new microcentrifuge tube and the pellets were discarded. Protein concentration was quantified for each processed lysate in accordance with the BioRad DC Protein Assay (BioRad) standard 96-well plate protocol. Following quantification, each sample was mixed with an equal volume 2 x Laemmli buffer (9.9 g/L Tris, 20 % glycerol, 10% SDS, 0.02% bromophenol blue) and 5 µL of a 3M dithiothreitol (DTT, BioShop) solution, then boiled at 96˚C for 5 minutes. Protein samples were either used in SDS-polyacrylamide gel electrophoresis or stored at -20˚C.

SDS-polyacrylamide gels (8-12%) were made as described by Sambrook and Russell (571), and cast using a Hoefer gel caster system (SE260). Depending on the experiment, between 10-20 µg of total protein for each protein sample was loaded per lane. Color Prestained Broad Range Protein Standard (NEB) was used as a protein ladder for all SDS-polyacrylamide gels. Gels were run in SDS running buffer (14.4 g/L glycine, 3 g/L Tris, 0.1% SDS) at 120 V until dye front ran off the end of the gel. Gels were transferred to polyvinylidene fluoride (PVDF) membranes (BioRad) using the BioRad Turbo Blot transfer system in accordance with the BioRad Trans-Blot Turbo RTA Transfer Kit. Transferred membranes were rinsed in Tris-buffered saline with Tween-20 (TBST, 8.8 g/L NaCl, 6 g/L Tris, 1% Tween-20 (Bioshop), pH 7.6) then blocked on a shaker at room temperature in blocking buffer (5% bovine-serum albumin (BSA, BioShop) in TBST) for one hour. Primary antibody was used in the concentration described in Table 2.5, diluted in the same 5% BSA blocking buffer used above or a 5% skim milk blocking buffer (5% skim milk powder (Carnation) in TBST). Membranes

were incubated in primary antibody overnight on a shaker at 4°C. The following day, membranes were washed four times, 5 minutes each, with TBST and subsequently incubated in secondary antibody as described in Table 2.5, on a shaker for 1 hour. Membranes were washed an additional four times, five minutes each, with TSBT. Membranes were then developed using Clarity Western ECL Substrate (BioRad) in accordance with supplier protocol. Images were acquired using a ChemiDoc Touch Imaging System (BioRad).

2.4.8. Core Genome Phylogeny

The core genome analysis was performed in 2,544 S. *enterica* genomes using SaturnV (v1.1.0; https://github.com/ejfresch/saturnV). To perform core genome phylogenetic analysis, two criteria were used for core gene inclusion. First, genes were considered core genes if they were identified in all genomes analyzed. Second, core genes for which one and only one ortholog in each of the other genomes was found (1:1 core genes) were included. Two genes were considered ortholog genes when their alignment covered more than 85% of their length and shared more than 50% of sequence identity. Core genes were aligned using Prank (v.150803) (572) and uninformative positions were removed with BMGE (v.1.12) (573). Finally, the phylogenetic tree was generated using FastTree based on 173,657 SNPs (v. 2.1.8; bootstraps: 1000; model: GTR) (574).

2.4.9. SspH Clustering and Multidimensional Scaling

The related proteins to the virulence factor SspH1 (UniProt accession number: D0ZVG2) were identified in 2,544 S. *enterica* genomes from the *Salmonella* Syst-

Table 2.5: Antibody list

Antibody	Buffer	Secondary	Dilution	Source
Actin-HRP[1]	5% Milk	None[2]	1:2000	Cell Signaling 5125S
PKN1	5% BSA	Mouse	1:1000	R&D Systems MAB6100
p-Akt Ser473	5% BSA	Rabbit	1:1000	Cell Signaling 9271S
Total Akt	5% BSA	Rabbit	1:1000	Cell Signaling 9272S
Anti-mouse HRP	Same as primary	N/A[3]	1:2000	Cell Signaling 7076S
Anti-rabbit HRP	Same as primary	N/A	1:2000	Cell Signaling 7074S

[1]Horseradish peroxidase
[2]No secondary incubation required
[3]Not applicable

OMICS isolate consortium using BLASTp (identity $\geq 50\%$ and coverage $\geq 85\%$). SspH1-related proteins were clustered with greater than 90% identity using Uclust (v1.2.22q) (575). Proteins were aligned using MUSCLE (v3.8.31) (576) and the alignment was imported and converted into a distance matrix by the R package SeqinR (v3.6-1) (577) using protein similarity.

Chapter 3: Impact of *Salmonella* contamination on microbial-related food recalls in Canada

A version of this section is section is published in *Journal of Food Protection,* 82(11): 1901-1908. (2019).

3.1. Introduction

Foodborne illnesses are a public health concern world-wide. In Canada, approximately 4 million foodborne illnesses are contracted annually, leading to an estimated 11,600 hospitalizations and 240 deaths (548). While the microbes behind many of these foodborne illnesses go unidentified, a group of 30 pathogens are believed to be mainly responsible. *Salmonella* is a substantial contributor to foodborne illness from this group and is the leading cause of hospitalizations linked to bacterial foodborne illness in Canada (578). *Salmonella* infections commonly produce mild and self-limiting disease and often go undiagnosed or undocumented. As a result, current estimates of *Salmonella* illness may not accurately reflect the true incidence of infection.

Like most foodborne illnesses, *Salmonella* are transmitted via the ingestion of contaminated or improperly prepared food. When *Salmonella* contamination is suspected in food products, food recalls are conducted to prevent the spread of foodborne infection. Manufacturers and distributors are prohibited from selling food products that violate section 4 of the Food and Drugs Act, such as microbial-contaminated foods, and are responsible for removing the products from sale and/or distribution (579). However, recall of unsafe food products was voluntary until the enactment in April 1997 of the Canadian Food Inspection Agency (CFIA) Act. The CFIA Act granted authority to the Minister of Agriculture and Agri-Food to order companies to recall food products that

were reasonably believed to pose a risk to the public. The CFIA Act also created the CFIA and established a framework to investigate and identify potentially harmful food products for recall (580).

The CFIA conducts food safety investigations in response to a variety of triggers to determine if a food recall is required. Triggers include a consumer complaint or illness outbreak linked to a specific food, a food test result or CFIA inspection result that indicates a potential health risk, a company-initiated recall, or a recall in another country (581). In response to the trigger, the CFIA-led food safety investigation aims to determine the specific unsafe food product, the area of distribution, and the agent responsible for the safety risk. If a potential health risk is identified, a health risk assessment is conducted by Health Canada to determine the likelihood that the food will cause illness and the potential duration and severity of illness (554). The CFIA then uses the health risk assessment to determine if a food recall is necessary and classifies the recall as high (class I), intermediate (class II), or low (class III) risk (552). Following the recall order, the manufacturer or distributor is responsible for recalling the unsafe food product from all of its accounts. The CFIA will continue to assist in the process through managing the implementation of the recall, publishing recall warnings, and verifying that the company has recalled products from retail shelves (581). The procedures used by CFIA-regulated manufacturers and distributors to conduct recalls of unsafe food are described in section 84 of the *Safe Food for Canadians Regulations* (582).

The United States Food and Drug Administration (FDA) has published numerous studies documenting food recall trends in the United States (555,583,584). These reports consistently showed microbial-related food recalls as a large contributor to all food recall

events. Additionally, the most recent report revealed *Salmonella* as the leading contributor to microbial-related food recalls. While the CFIA has existed for the entirety of the 21st century, no study has consolidated microbial-related food recall data from Canada to examine annual recall trends. I hypothesize that *Salmonella* is a major contributor to microbial-related food recalls in Canada. Therefore, the objective of this work was to measure the contributions of *Salmonella* relative to all microbial-related food recalls in Canada under the jurisdiction of the CFIA for years 2000 to 2017.

3.2. Results

3.2.1. Microbial-Related Food Recall Events and Recalled Food Products, Canada 2000-2017

From January 1st, 2000 through December 31st, 2017, the CFIA recorded a total of 2094 recall events and 10432 recalled food products (Table 3.1). Years 2011-2017 accounted for a majority of the recall events (51.5%) and recalled food products (52.9%) from the entire time period analyzed, with 2012 leading all years in both categories. The data from Table 3.1 is visualized in Figure 3.1A. To analyze microbial-related food recalls from this period, I classified recall events and recalled food products based on food type, the area of concern, and the microbiological contaminate involved.

The CFIA uses industry codes to categorize food recalls into related product types. These product types are broad and non-specific compared to the classification system used by the United States FDA. Therefore, I further categorized the microbial-related food recalls using United States FDA product types (Table 3.2). From 2000-2017, at least one recall event occurred for each of the 35 product categories used to classify the microbial-related food recalls. The product categories with the greatest number of recalls

Table 3.1: Number of food recall events and products recalled for microbial contamination by year, 2000 through 2017, by the Canadian Food Inspection Agency.

	Recalls	
Year	No. of events	No. of products
2000	37	70
2001	88	278
2002	82	165
2003	45	80
2004	64	289
2005	81	207
2006	80	475
2007	126	338
2008	108	674
2009	161	1005
2010	144	1337
2011	117	573
2012	241	1748
2013	153	534
2014	152	747
2015	139	631
2016	135	485
2017	141	796
Total	2094	10432

events were 'meat, meat products, poultry' (25.1%), 'fishery/seafood products' (12.3%), 'nuts and edible seeds' (10.9%), and 'cheese, cheese products' (10.2%). The product category 'meat, meat products, poultry' also accounted for largest number of recalled products (31%), followed by 'nuts and edible seeds' (11.6%), 'cheese, cheese products' (9%), and 'vegetables (leafy greens)' (8.2%). For the purposes of recall analysis, I considered product types with more than 50 recall events during the surveyed period to be the most contaminated food items. These product types were examined further in section 3.2.4.

A.

B. **C.**
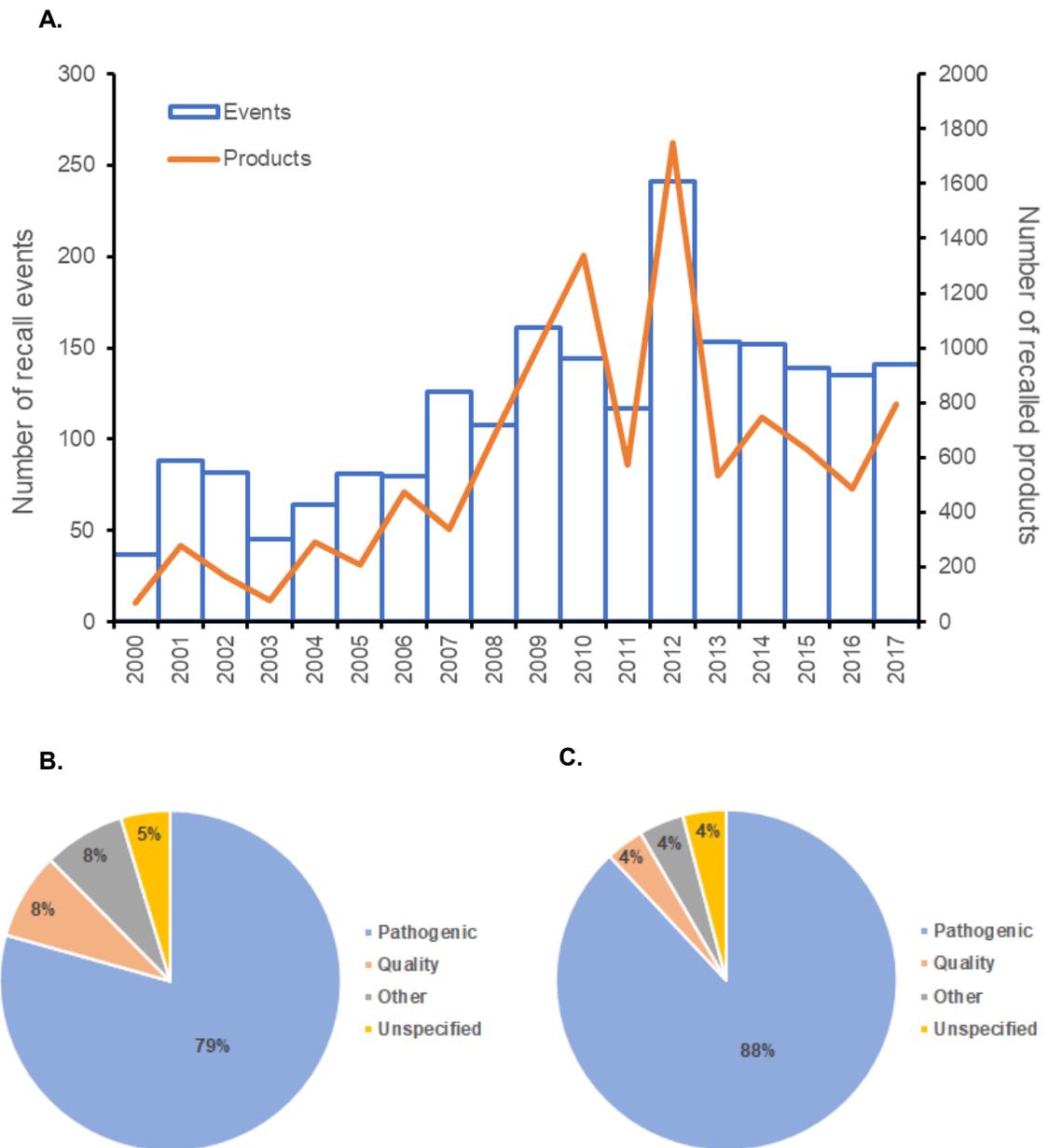
Figure 3.1: Microbial-related food recall events and recalled products, 2000-2017.
(A) Annual microbial-related food recall events (bars, left y-axis) and recalled product (line, right y-axis) totals reported by the CFIA, 2000-2017. (B) Percentages of microbial-related recall food recall events stratified by microbiological area of concern (defined in Table 2.1). The category "Other" was separated from "Unspecified" due to its relatively larger contribution to microbial-related food recalls. (C) Percentages of microbial-related recalled food products stratified by microbiological area of concern.

Table 3.2: Recall events and recalled food products, by product type, resulting from microbial contamination, years 2000-2017, per Canadian Food Inspection Agency.

Product Type	No. of recall events	No. of products recalled
Alcoholic beverages	8	12
Animal food	1	3
Baby (infant and junior) food	6	41
Bakery products (mixes, and icings)	37	158
Beverage bases	2	2
Candy without chocolate	6	31
Cereal preparations, breakfast foods	2	5
Cheese, cheese products	213	936
Chocolate and cocoa products	18	45
Coffee and tea	12	18
Dietary conventional foods	10	43
Dressings and condiments	17	42
Eggs, egg products	18	41
Filled milk, imitation milk products	4	9
Fishery/Seafood products	257	782
Food sweeteners (nutritive)	3	11
Fruit and fruit products	114	379
Ice cream and related products	10	71
Macaroni and noodle products	4	10
Meat, meat products, poultry	525	3234
Milk, butter, and dried milk products	43	116
Multiple foods dinners (sauces)	106	681
Nuts and edible seeds	229	1214
Prepared salad products	25	317
Snack food items	17	46
Soft drinks and water	25	54
Soups	16	42
Spices, flavours, and salts	98	512
Vegetable oils	2	3
Vegetable protein product	8	48
Vegetables, vegetable products	123	391
Vegetables (leafy greens)	60	858
Vegetables (roots, tuber, fungi)	25	63
Vegetables (sprouts)	34	72
Whole grains, milled grain products	16	142
Total	2094	10423

The CFIA categorizes microbial-related food recalls into multiple areas of concern listed in Table 2.1. During the time period surveyed, pathogenic bacteria accounted for

the largest number of food recall events and recalled food products, 79% and 88%, respectively, followed by food quality issues related microbial contamination, 'other', and 'unspecified' (Figure 3.1B-C). While microbial-related food recalls related to 'other' areas of concern are typically listed within 'unspecified', the two categories were separated as the total number recalls associated with 'other' was larger than the total for 'unspecified'. The four areas of concern categories are classified further by the pathogen, toxin, or condition responsible for the microbial-related food recall (Table 3.3). Among the 2094 food-related recall events, 522 (24.9%) involved contamination with *Salmonella* spp., 508 (24.3%) involved contamination with *Listeria monocytogenes*, and 266 (12.7%) involved contamination with *E. coli* O157:H7. While *Salmonella* was responsible for the largest number of contaminated food recall events, most of its recall events (77.6%) were categorized as class II. *L. monocytogenes* accounted for the largest number of class I food-related recalls (37.5%), followed by *E. coli* O157:H7 (29.5%), and *C. botulinum* (14%). *L. monocytogenes* was also responsible for the largest number of recalled contaminated food products from 2000-2017, followed by *E. coli* O157:H7 and *Salmonella*. Class III food-related recalls, the lowest risk classification, were primarily associated with recalls related food quality issues (40.7%); although, 'other' (19.4%) and *L. monocytogenes* (10.9%) were substantial contributors as well.

3.2.2. Microbial-Related Food Recall Events by Product Type, Canada 2000-2017

Considering *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 are responsible for a majority of the reported microbial-related food recalls, I characterized their associated recall events by product type to examine pathogen-specific food product

Table 3.3: Number and classification of food-related recall events and products recalled for microbial contamination, by pathogen, or toxin, or condition, years 2000 through 2017 by the Canadian Food Inspection Agency.

Pathogen, toxin, or condition	No. of events				No. of products recalled
		Classification			
	Total	I	II	III	
Salmonella	522	112	405	5	2325
Listeria monocytogenes	508	310	157	41	2942
E. coli O157:H7	266	244	21	1	2577
Clostridium botlinum	139	116	14	9	440
Other[1]	126	12	41	73	346
Non-O157:H7 *E. coli*	96	6	59	31	300
Mold	82	–[2]	2	80	174
Staphylococcus aureus	63	4	56	3	142
Container integrity	56	1	47	8	315
Undetermined	42	1	11	30	114
Vibrio parahaemolyticus	33	2	19	12	196
Yeast	33	–	–	33	82
Marine biotoxin	30	9	18	3	80
Coliforms	23	–	5	18	44
Generic *E. coli*	17	–	12	5	19
Aerobic colony count	17	–	–	17	58
Pseudomonas aeruginosa	12	–	11	1	23
Bacillus cereus	8	1	5	2	19
Cyclospora	6	3	1	2	20
Multiple pathogens	6	3	2	1	195
Shigella flexneri	5	3	2	–	10
Clostridium perfringens	4	–	3	1	11
Total	2094	827	891	376	10432

[1]Includes norovirus, hepatitis A virus, spoilage, and unspecified pathogenic bacteria.
[2] – no data.

trends. (Table 3.4). From 2000 through 2017, *Salmonella*-related food recalls affected a

wider range of product types (26 out of 35) relative to *L. monocytogenes* (20 out 35) and

E. coli O157:H7 (9 out of 25). *E. coli* O157:H7-related recall events were primarily

Table 3.4: Number of food-related recall events by industry and pathogen, years 2000 through 2017, by Canadian Food Inspection Agency.

Industry	E. coli O157:H7	Listeria monocytogenes	Salmonella
Alcoholic beverages	−[1]	−	−
Animal food	−	−	1
Baby (infant and junior) food	−	−	1
Bakery products (mixes, and icings)	−	3	4
Beverage bases	−	−	−
Candy without chocolate	−	1	−
Cereal preparations, breakfast foods	−	−	1
Cheese, cheese products	6	105	11
Chocolate and cocoa products	−	−	16
Coffee and tea	−	−	11
Dietary conventional foods	1	3	2
Dressings and condiments	−	−	−
Eggs, egg products	−	5	10
Filled milk, imitation milk products	−	3	−
Fishery/Seafood products	−	46	17
Food sweeteners (nutritive)	−	−	−
Fruit and fruit products	4	11	29
Ice cream and related products	−	6	1
Macaroni and noodle products	−	−	−
Meat, meat products, poultry	230	181	32
Milk, butter, and dried milk products	−	8	1
Multiple foods dinners (sauces)	1	51	7
Nuts and edible seeds	11	13	194
Prepared salad products	−	18	2
Snack food items	−	1	13
Soft drinks and water	−	−	−
Soups	−	−	1
Spices, flavours, and salts	−	−	91
Vegetable oils	−	−	−
Vegetable protein product	4	1	2
Vegetables, vegetable products	−	39	22
Vegetables (leafy greens)	7	4	32
Vegetables (roots, tuber, fungi)	−	8	1
Vegetables (sprouts)	2	1	19
Whole grain, milled grain products	−	−	1
Total	266	508	522

[1] – no data.

associated with the 'meat, meat products, poultry' category (86.5%) with a small contribution from 'nuts and edible seeds' (4.1%). A majority of *L. monocytogenes*-related recall events were associated with 'meat, meat products, poultry' (35.6%) and 'cheese, cheese products' (20.7%) with smaller contributions from 'multiple foods dinners (sauces)' (10%) and 'fishery/seafood product' (9.1%). While *Salmonella*-recall events were linked to the greatest range of product types, 'nuts and edible seeds' (37.2%) and 'spices, flavours, salts' (17.4%) accounted for a majority if its recalls.

3.2.3. Major Microbial-Related Food Recall Events, Canada 2000-2017

In 2008, *L. monocytogenes* was responsible for the recall of hundreds of contaminated food products in Canada due to the outbreak of listeriosis linked to the consumption of deli meats (585). I identified major microbial contaminated food recalls from 2000-2017 that were similar in magnitude to the *L. monocytogenes* recall in 2008 and classified the events by pathogen (Table 3.5). A total of 28 major microbial-related food recall events occurred between 2000-2017. *L. monocytogenes* accounted for the largest number of major recalls during this period with twelve, followed by *E. coli* O157:H7 and *Salmonella* both with six. With regards to single major food recalls, *E. coli* O157:H7 was responsible for the largest food product recall in 2012 for contamination of raw beef, followed by *Salmonella* for the contamination of peanuts in 2009. Only twelve of the major microbial-related food recalls resulted in reported foodborne illness with the largest outbreak (82 cases) linked to *Vibrio parahaemolyticus* contaminated oysters in 2015 followed by *Salmonella* contaminated sprouted chia seed powder (63 cases) in 2014.

Table 3.5: Major recalls of food products (minimum 50 product recalls) by pathogen and product type, years 2000 through 2017, per Canadian Food Inspection Agency.

Pathogen	Product recalled	No. of recalled products	Cases	Year	Ref.
E. coli O157:H7	Spinach	289	1	2006	(586)
Salmonella	Sesame seeds	62	-[1]	2007	(587)
E. coli O157:H7	Raw beef	95	45	2007	(588)
Listeria monocytogenes	Cheese	51	-	2008	(589)
L. monocytogenes	Deli meats	275	57[2]	2008	(585)
L. monocytogenes	Sandwiches	95	57[2]	2008	(585)
Salmonella	Peanuts	682	1	2009	(590)
L. monocytogenes	Cheese	152	-	2010	(591)
Salmonella	Herbs	110	-	2010	(592)
L. monocytogenes	Deli meats	90	-	2010	(593)
Multiple pathogens[3]	Ready-to-eat cooked meat	182	-	2010	(594)
Salmonella	Hydrolyzed vegetable protein	207	-	2010	(595)
E. coli O157:H7	Romaine	132	-	2010	(596)
L. monocytogenes	Cheese	68	1	2011	(597)
L. monocytogenes	Mixed greens	89	-	2012	(598)
E. coli O157:H7	Raw beef	1363	18	2012	(599)
L. monocytogenes	Potato, coleslaw, and pasta salads	176	-	2013	(600)
Salmonella	Sprouted chia seed powder	60	63	2014	(601)
L. monocytogenes	Cheese	221	-	2014	(602)
L. monocytogenes	Stone fruit	105	-	2014	(603)
Salmonella	Garlic powder	89	-	2015	(604)
Vibrio parahaemolyticus	Oysters	137	82	2015	(605)
E. coli O157:H7	Raw beef	64	-	2016	(606)
L. monocytogenes	Salad Kit	57	14	2016	(607)
E. coli O157:H7	Multiple raw meat types	114	-	2017	(608)
L. monocytogenes	Mixed vegetables	129	-	2017	(609)
E. coli (Non-O157)	Raw baked goods	90	30[4]	2017	(610)
E. coli (Non-O157)	Flour	115	30[4]	2017	(610)

[1] – no data.

[2] Reported cases of listeriosis were group together for both deli meats and sandwich recalls.

[3] *L. monocytogenes* and *Salmonella* both found in ready-to-eat cooked meat

[4] Reported cases of foodborne illness linked to *E. coli* O121 were group together for both contaminated raw baked goods and flour.

3.2.4. Decade Analysis of *Salmonella*-Related Food Recall Events, Canada 2000-2017

To examine trends in *Salmonella*-related food recalls, I first compared all microbial-related recall event totals from 2000-2009 (first decade) to the event total from the 2010-2017 (second decade). From a broad perspective, the 2010-2017 period reported 350 more recall events than the previous decade (Table 3.1), even with the absence of recall event totals for the final two years of the decade. This increase in recall events from the first decade to the second is reported for each of the most contaminated food products (Figure 3.2A). I measured the relative increase of recall events for the most contaminated food products, identified in section 3.2.1, to further quantify the recall-related changes between the surveyed periods (Figure 3.2B). The categories 'spices, flavours, salts', 'vegetables (leafy greens)', 'vegetables, vegetable products' and 'cheese, cheese products' increased by more than half their recall events between the two periods, whereas the remaining categories reported moderate increases from their first decade totals.

Next, I compared *Salmonella*-related recall events between the two decades for the most contaminated food products. To account for the changes in recall reporting between the two decades, I normalized *Salmonella*-related recall event totals by the total number of microbial-related recall events for each product type and decade. The result is a measure of *Salmonella*-related recall events for each of the most contaminated food products relative to the total number of recall events per product type in each decade (Figure 3.2C). These relative recall event totals demonstrate that *Salmonella* is responsible for majority of the recall events involving 'nuts and edible seeds' and 'spice,
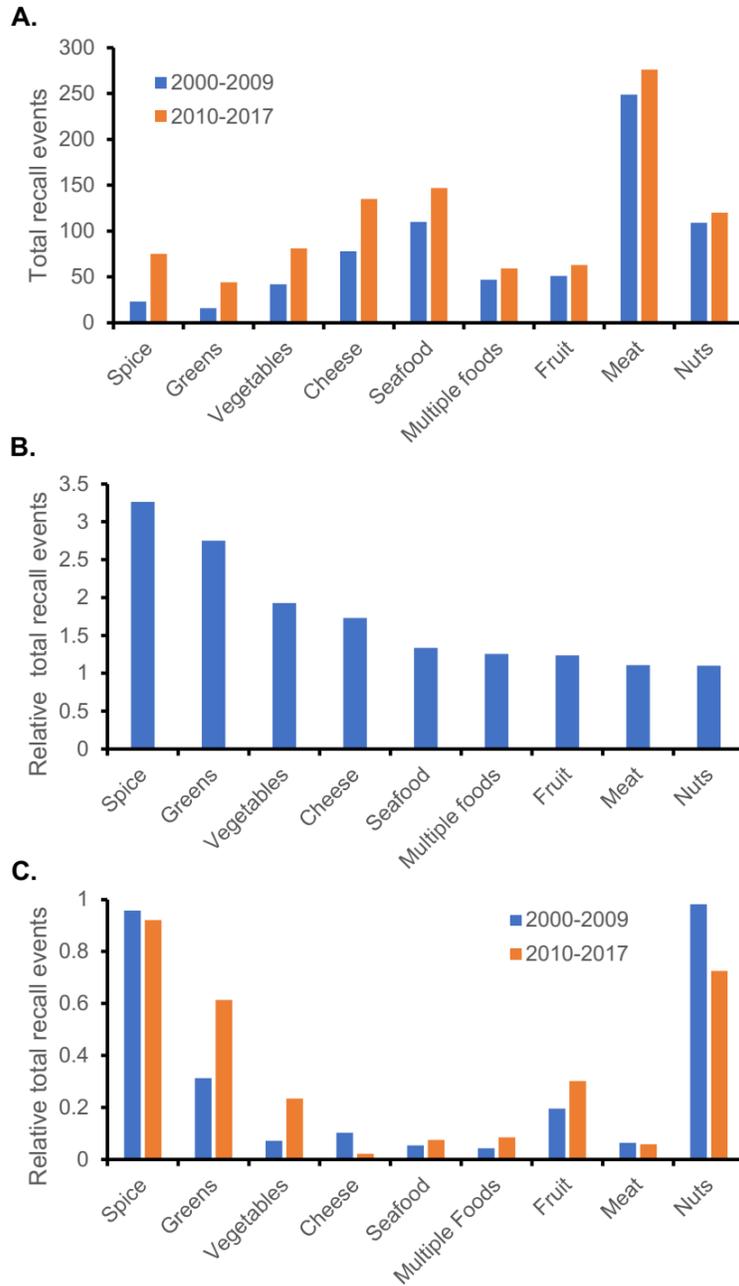
Figure 3.2: Decade analysis of *Salmonella* impact on microbial-related food recalls.
(A) Microbial-related food recall event totals for most contaminated food products (minimum 50 recall events, 2000-2017) separated by decade (2000-2009 (blue), 2010-2017 (orange)). (B) Relative microbial-related food recall event totals (2010-2017 total/2000-2009 total) for most contaminated food products. (C) Normalized *Salmonella*-related food recall event totals for most contaminated food products (# of *Salmonella*-food product recall events/total # of food product recall events) separated by decade (2000-2009 (blue), 2010-2017 (orange)).

flavours, salts'. *Salmonella*-related recalls account for approximately 90% of all 'spice, flavours, salts' recall events in both the first and second decade, with a small decrease observed in the second decade. *Salmonella* was also responsible for nearly all 'nuts and edible seeds' recall events in first decade at 98% but dropped to 73% in the second decade. While the relative recall event totals decreased for these two categories between decades, *Salmonella*-related recalls increased for several other product types. The largest increases in relative *Salmonella*-related recall event totals between decades are reported for 'vegetables (leafy greens)' (31.3% to 61.4%) followed by 'vegetables, vegetable products' (7.1% to 23.5%) and 'fruit and fruit products' (19.6% to 30.2%). *Salmonella*-related recall events contributed minimally to the remaining most contaminated food product categories.

3.3. Discussion

In this section, I reviewed food-related recalls associated with microbial contamination from 2000-2017 classified by the CFIA to measure the contributions of *Salmonella* to food recalls in Canada. The CFIA has previously released reports analyzing food recall incidents as well as food safety investigations, but both reports analyze a shorter time period and do not focus on microbial contamination to the extent of this study (611,612). My analysis revealed that from 2000-2017, a total of 2094 microbial-related food recall events were recorded in Canada and that, until 2006, the annual number of these events remained below 100 (Table 3.1). The average annual total of microbial-related food recall events increased from 87 events per year in the 2000s (2000-2009) to 153 in 2010s (2010-2017). This increase in reported microbial-related recall events in the 2010s by 75% may represent an increased frequency of microbial

contamination of food products but could also be attributed to the technical improvement of foodborne pathogen diagnostics and enhanced efforts in food safety investigations and outbreak surveillance (613,614).

Although many pathogens are responsible for food recalls, *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 collectively accounted for 61% of all microbial-associated recall events from 2000-2017 in Canada (Table 3.3). *Salmonella* was associated with the highest number of recall events during this time, but a majority the recalls were categorized as class II indicating an intermediate risk for adverse health consequences. The high number of recall events associated with *Salmonella* contamination coincides with a high *Salmonella* hospitalization incidence estimate, reported by Thomas *et al.* (548). While *Salmonella* is associated with the highest hospitalization rate among foodborne bacteria, both *L. monocytogenes* and *E. coli* O157:H7 are responsible for a greater death rate relative to illness incidence (548). The lethality associated with *E. coli* O157:H7, and to a greater extent, *L. monocytogenes* is reflected in recall event classification, as a majority of *L. monocytogenes* and *E. coli* O157:H7 contaminated food products were categorized as class I (high risk). Aside from food recall events, *Salmonella*, *Listeria*, and *E. coli* O157:H7 collectively accounted for 77% of all recalled food products due to microbial contamination from 2000-2017. The high proportion of recalled food products associated with *Salmonella*, *L. monocytogenes*, and *E. coli* O157:H7 contamination is the result of the many major recall events (minimum 50 products recalled) linked to these foodborne pathogens (Table 3.5).

Microbial contamination can affect a variety of food products, as examined by this work. I categorized the microbial-contaminated food recall data using 34 different

food product types in accordance to the United States FDA defined product codes, with one addition (sprouts). From the 35 different product types, nine categories reported more than 50 recall events over the surveyed period (Table 3.2). Consequently, I labelled these nine food categories as the most contaminated food items. The categories 'meat, meat products, poultry', 'nuts and edible seeds', and 'cheese, cheese products' were among the top product types for both total recall events and number of products recalled. Both *L. monocytogenes* and *E. coli* O157:H7 are mainly responsible for the recall total reported for 'meat, meat products, poultry' as they are associated with infamous recalls of deli meats and raw beef, respectively (585,599). *L. monocytogenes* contamination also accounts for a majority of 'cheese, cheese products' recalls, which is expected considering its well-documented history of contaminating dairy products (615). Regarding 'nuts and edible seeds', *Salmonella* contamination is responsible for most of the recalled food products due to a peanut contamination event that affected both the United States and Canada (590,616). The category 'fishery/seafood products' was also a leading contributor to microbial-related recall events. Unlike the previous categories, 'fishery/seafood products' were associated with a greater diversity in microbial contamination, as twelve different pathogens, toxins, and conditions were linked to their recalls.

Several other product types recorded substantial totals of microbial-related recall events including three produce categories: 'vegetable, vegetable products', 'vegetables (leafy greens', and 'fruit and fruit products' (Table 3.2). *L. monocytogenes* and *Salmonella* are largely responsible for the recalls totals reported for these categories as they are both linked to outbreaks associated with contaminated produce in North America

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(586,617–619). The category 'multiple foods dinners (sauces)' also recorded a high total of recall events, of which a large portion are associated with ready-to-eat (RTE) meals. *L. monocytogenes*-contamination accounts for nearly a majority of 'multiple foods dinners (sauces)' recalls, which is expected given its widely documented record of contaminating RTE meals (620,621). The final product type with substantial recall event totals, 'spices, flavours, salts', is almost exclusively associated with *Salmonella*-related recalls. This predominance is reported globally and is often linked to the desiccation tolerance of *Salmonella* (622–624).

Over the course of the two decades examined by this work, the CFIA facilitated 26 microbial-contaminated food recalls that we designated as major recalls (Table 3.5). The most well-known major Canadian recalls among the 26 reported include the *L. monocytogenes*-contaminated deli meats in 2008 (585) and the *E. coli* O157:H7 contamination of raw beef in 2012 (599). Both contamination incidents were a result of poor sanitation practices during production and manufacturing and resulted in documented cases of human illness and deaths (625,626). The second largest recall in Canadian history involved *Salmonella* contaminated peanut products from the United States. While only a single illness was linked to contamination in Canada, a more severe outbreak occurred in the United States with 714 reported cases and nine deaths (590). In addition to these three foodborne outbreaks, I was interested in determining how often major recalls were associated with transmission of foodborne illness. Among the 26 major recall events identified, twelve were linked to reported illnesses. Four of these twelve recalls were associated with *L. monocytogenes* contamination, while three *E. coli* O157:H7 and two *Salmonella*-related major recall events reported cases foodborne

illness. Within the last six years, three major recalls have all accounted for at least 30 documented cases of foodborne illness. Two of these recent major recalls, *Salmonella* contamination of sprouted chia seed powder and *E. coli* O121 of flour and raw baked goods, remain without an identified source of the contamination (610,627). The third recent major recall involved *Vibrio parahaemolyticus* contaminated oysters resulting in 82 reported cases of illness, the highest among all major recall events (605).

Trends in foodborne illness can change dramatically from decade to decade as observed with the decrease in botulism cases from 1985 through 2005 in Canada (628). I examined food recall events and products associated with microbial contamination from 2000-2009 and 2010-2017 to identify potential trends with foodborne pathogens. *E. coli* O157:H7 contamination demonstrated the highest increase between decades in both microbial-related recall events (74 for 2000 to 2009, 192 for 2010 to 2017) and recalled contaminated food products (554 for 2000-2009, 2023 for 2010 to 2017). Both increases in relative recall events and recalled food products were primarily attributed to a substantial number of recalls for raw beef in 2012 that affected over 1300 products (599). Similar to *E. coli* O157:H7, recall events attributed to either *L. monocytogenes* or *Salmonella* contamination increased, specifically by 70 events for each (219 to 289 for *L. monocytogenes* and 226 to 296 for *Salmonella*). However, the increase in total recalled food products associated with *L. monocytogenes* between the two decades (939 for 2000-2009, 2003 for 2010-2017) was much greater than that for *Salmonella* (1129 for 2000-2009, 1196 for 2010-2017). From 2010-2017, *L. monocytogenes* accounted for twelve major food recalls, while *Salmonella* was associated with six (Table 3.5). Even though similar increases in recall events were reported for both *L. monocytogenes* and

Salmonella, more *L. monocytogenes* contaminated food products were recalled due to a higher number of major recall events.

In addition to examining general pathogen recall trends, I analyzed *Salmonella*-specific recalls by the most contaminated product types for each decade. *Salmonella* was responsible for nearly all food recalls from the 'spices, flavours, salts' category in both decades (Figure 3.2C). The microbial-related recall event total for this category also increased by approximately three and a half fold in the second decade relative to the first decade (Figure 3.2B). Therefore, the substantial increase in total recall events reported for the 'spices, flavours, salts' category is primarily attributed to *Salmonella* contamination. Unlike 'spices, flavours, salts', the recall event total for the 'nuts and edible seeds' category in the second decade was on par with the previous decade total. Additionally, the relative percentage of *Salmonella*-related 'nuts and edible seeds' recall events reported in the second decade decreased considerably, indicating an increase in recalls linked to other microbial contaminates. Increases in relative *Salmonella*-related recall event totals were observed for three product types in the second decade: 'vegetables (leafy greens)', 'vegetables, vegetable products', and 'fruits and fruit products'. Microbial-related recall event totals also increased for these product types in the second decade, with substantial increases observed in the 'vegetable (leafy greens)' and 'vegetables, vegetables products' categories. Altogether, this increase in *Salmonella*-related produce recalls highlights the growing concern of *Salmonella* outbreaks linked to the consumption of contaminated fruits and vegetables (586,619).

The work presented in this section on microbial-related food recalls reported in Canada fills a gap in recall analysis that has remained vacant since the CFIA initiated

recall data documentation in 1997. I report that *Salmonella* is a leading contributor to microbial-related food recall events in Canada and is responsible for contaminating the widest range of product types relative to all other microbial-contaminates. *Salmonella* contamination is chiefly responsible for a majority of food recalls related to nuts/edible seeds and spices and is increasingly contributing to recalls associated with fruits and vegetables. Collectively, these findings demonstrate that *Salmonella*-contamination is a considerable threat to food safety in Canada.

Chapter 4: Protozoa as a Screening Model for *Salmonella* Virulence

4.1. Introduction

The genus *Salmonella* is incredibly diverse as demonstrated by the spectrum of host range and pathogenicity prevalent across more than 2600 serovars. While some serovars are host restricted, such as *Salmonella* ser. Typhi and Gallinarum, others exhibit unrestrained host tropism (29). A similar range of diversity is also observed in *Salmonella* pathogenicity as some serovars are more likely to produce invasive infection than others (629). Despite advancements in genomic characterization of *Salmonella*, correlation of serovar-specific genetic analysis to pathogenic consequences is limited. Furthermore, genetic characterization of *Salmonella* pathogenesis is largely focused on the serovar Typhimurium as a model of non-typhoidal disease (630). *Salmonella* ser. Typhimurium, however, is not solely responsible for all *Salmonella* infections in humans as many serovars contribute to foodborne illness (545,546). Studies on *Salmonella* ser. Typhimurium pathogenesis do not reflect the range of pathogenicity present across the genus due to the genomic heterogeneity of serovars (547). Therefore, additional research is required to connect the genetic diversity of *Salmonella* serovars to pathogenic markers to improve mitigation strategies for *Salmonella* contamination and transmission.

The genetic diversity across the genus *Salmonella* also transcends antigenic classification as varying degrees of virulence are observed between isolates from the same serovar (631–633). While serotyping provides antigenic information important for surveillance, it does not accurately indicate strain pathogenicity. Banding pattern subtyping methods such as pulsed-field gel electrophoresis (PFGE) and multiple locus variable number of tandem repeats analysis (MLVA) possess enhanced discriminatory

power relative to serotyping, but classify isolates based on generalized genetic characteristics that provide minimal insight into pathogenicity (634,635). The greatest subtyping discriminatory power lies with whole genome sequencing (WGS) analysis as it permits single nucleotide resolution comparison between isolates (636). Moreover, combining WGS with comparative genomics can identify differences in genetic elements that dictate variations in serotype (637,638), antimicrobial resistance (639,640), and virulence (641,642). However, identification of these genetic predictors is dependent on correlating WGS analysis with strain-specific virulence phenotypes.

Several groups have developed predictive models of virulence based on WGS and virulence correlation analysis in both *L. monocytogenes* and *Salmonella* isolates (643,644). While these studies demonstrate the potential of a virulence predictive model, they are limited by their inclusion of only a single measure of bacterial pathogenicity. Regarding *Salmonella*, optimal predictive modeling requires multiple parameters to effectively evaluate virulence, including host range, internalization, intracellular survival, and disease progression. The *Salmonella* Syst-OMICS project, led by Genome Canada, applied this multifaceted approach to identify genetic predicators of *Salmonella* pathogenicity through combining WGS analysis of 4500 clinical and environment isolates with infection data from multiple virulence screening models (645). These models include screenings with human epithelial cells, human macrophages, iv inoculated mice, and a protozoan infection model. My doctoral research contributed to the *Salmonella* Syst-OMICS project as I was responsible for developing the protozoan screening model to evaluate the virulence of *Salmonella* isolates.

In general, predation by protozoa has a significant effect on bacterial populations in the environment; however, some bacteria have acquired factors to evade predation. These factors are believed to have evolved in the context of continuous bacterial interactions with bacterivorous protozoa before animal forms of life emerged (646). Factors that inhibit or impair protozoa predation have been shown to also play an important role in the survival and persistence of foodborne pathogens outside the host cell, and in the environment. For example, spore formation in *Bacillus subtilis* provides resistance to both *Tetrahymena* digestion and harsh environmental conditions (647,648). Additionally, *Vibrio cholera* employs a T6SS to defend against amoeba and to kill other bacteria in close proximity (649). A portion of these factors may also contribute to survival within human hosts due to similarities in bactericidal processes used by both protozoa and macrophages (148,149,650). As a foodborne human pathogen, *Salmonella* encodes a variety of virulence factors that may have evolved as survival tools against bacterivorous protozoa. Several studies have confirmed that virulence factors associated with intracellular survival in mammalian hosts are also important in establishing resistance to protozoan predation (162,169,181). However, the protozoan models examined in these studies have not been used to characterize the virulence of *Salmonella* isolates. Therefore, I hypothesize that a protozoan infection model can distinguish between virulent and avirulent *Salmonella* isolates. The objective of this study was to develop a protozoan screening model that could distinguish between *Salmonella* isolates with varying virulence phenotypes. The work presented in this section evaluated the screening potential of eight different protozoa in distinguishing between well-documented virulent and attenuated *Salmonella* strains (Figure 4.1).
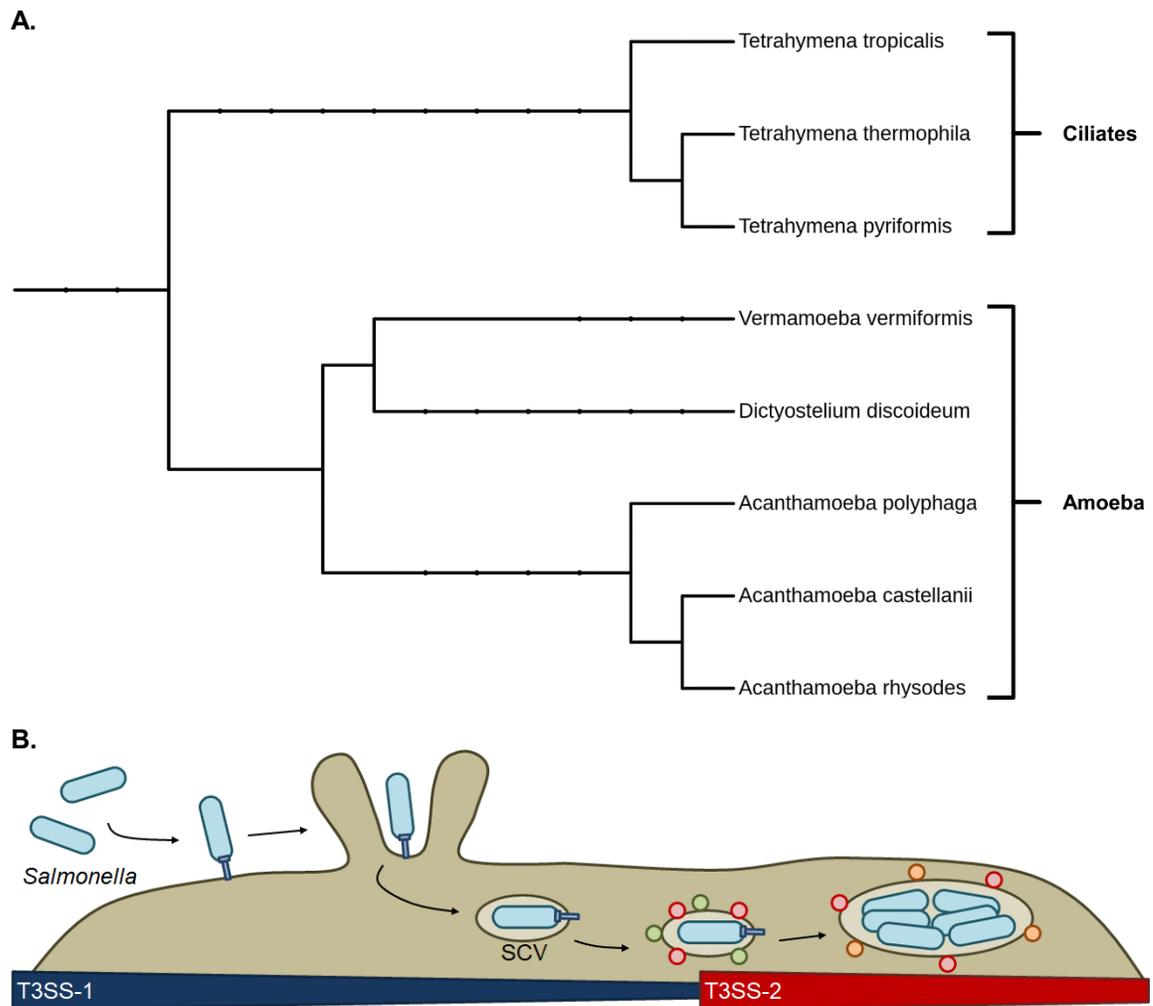
Figure 4.1: Bacterivorous protozoa and *Salmonella* virulence.
(A) Bacterivorous protozoa are eukaryotic unicellular organisms that exhibit predatory behaviour toward smaller organisms in their environment, such as bacteria. Two major protozoa groups that prey on bacteria include ciliates and amoebae. The species listed in the cladogram represent known bacterivorous protozoa that were used to screen for *Salmonella* virulence. The cladogram was created using https://phylot.biobyte.de. (B) *Salmonella* encodes two type three secretion systems, T3SS-1 and T3SS-2. T3SS-1 contributes to the internalization and SCV formation, while T3SS-2 contributes to SCV maturation and intracellular replication. Both T3SSs contribute to mammalian infection, but their contributions to protozoan infection are less characterized. To evaluate their contributions, all eight protozoan species were evaluated for their ability to distinguish between the *Salmonella* ser. Typhimurium parent strain SL1344 and a double mutant derivative defective for both T3SSs (*ΔinvAΔsseB*). InvA is an essential component to the T3SS-1, while SseB is required for proper functioning of the T3SS-2.

4.2. Results

4.2.1. *Salmonella* and Protozoa Survival in Protozoan Grazing Assay

Several studies have evaluated bacterial resistance to protozoan predation through co-culturing experiments in non-nutrient buffer (162,169,179). These experiments involve sampling buffer over the course of co-culturing to measure levels of bacterial consumption in response to protozoan grazing. I performed similar protozoan grazing experiments using six different species, three amoeba and three ciliates, to evaluate predation resistance between two *Salmonella* ser. Typhimurium strains, the laboratory strain SL1344 and a double mutant derivative Δ*invA*Δ*sseB* (Figure 4.2). InvA is component of the T3SS-1 and contributes to *Salmonella* internalization and SCV formation (651), whereas SseB is a subunit of the T3SS-2 translocon and facilitates SCV maturation and intracellular replication (652). Given that a functional T3SS-2 is required for replication in macrophages (653,654), an effective protozoan screening model for *Salmonella* virulence should distinguish between wild type *Salmonella* and the Δ*invA*Δ*sseB* mutant.

Grazing assays performed in all amoeba and ciliates displayed unremarkable differences in predation when comparing SL1344 measurements to Δ*invA*Δ*sseB* levels (Figure 4.3). The ciliates from the genus *Tetrahymena* consumed SL1344 and Δ*invA*Δ*sseB* equally as bacterial enumeration levels were similar at both 1- and 24-hours post inoculation. Conversely, the amoeba *Vermamoeba vermiformis* and *Acanthamoeba castellanii* displayed an inability to control either SL1344 or Δ*invA*Δ*sseB* growth as both strains increased by one thousand-fold between the 1-hour and 24-hour time points.
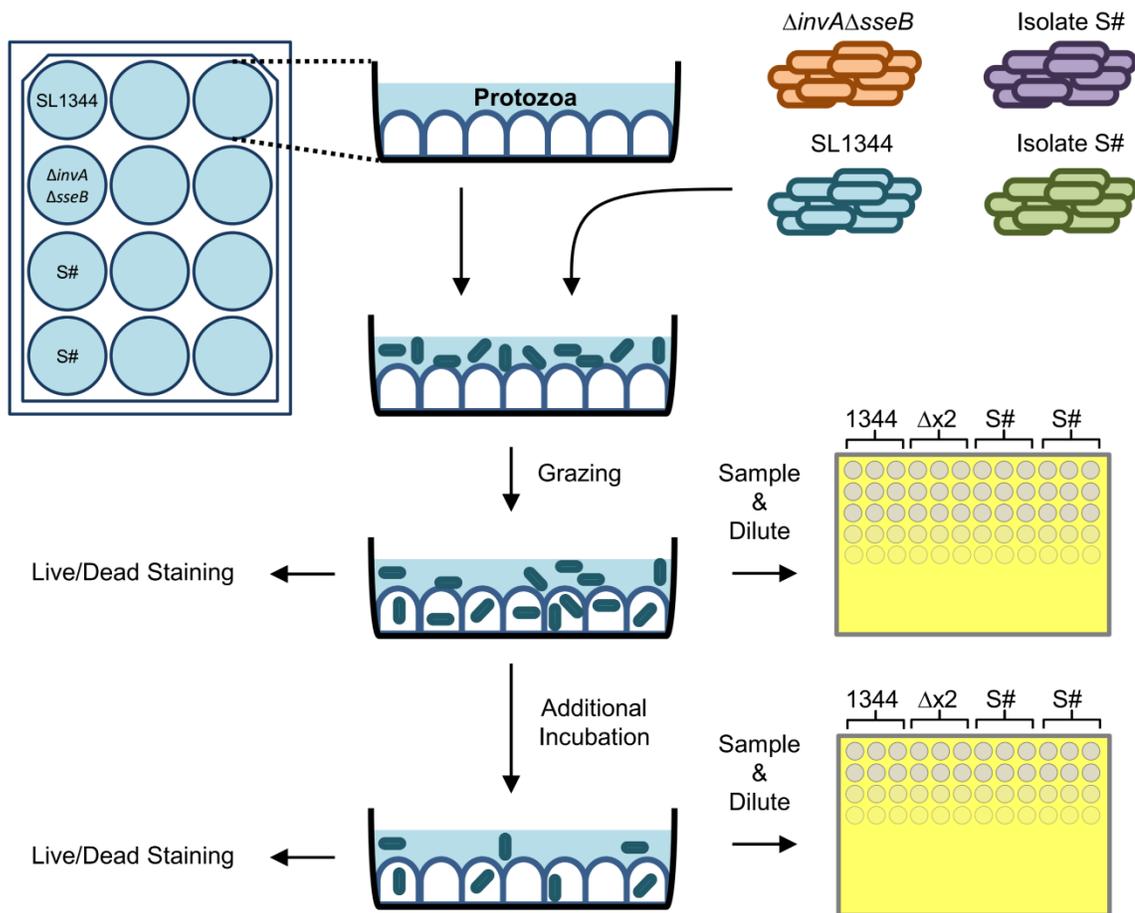
Figure 4.2: Evaluation of *Salmonella* survival and protozoa replication using a protozoan grazing assay.

A protozoan grazing assay is used to evaluate survival of *Salmonella* and replication of protozoa. The laboratory *Salmonella* ser. Typhimurium strain SL1344 and a double mutant derivative, Δ*invA*Δ*sseB*, are used as positive and negative control strains for *Salmonella* virulence, respectively. Wells seeded with protozoa in species-specific buffer are inoculated with control strains and *Salmonella* isolates at a species specific MOI (*Tetrahymena* = 1000, *Dictyostelium* = 100, *Acanthamoeba* & *Vermamoeba* = 10) and incubated at 30°C (22°C for *Dictyostelium*). Aliquots are taken from wells at 1- and 24-hours post infection for dilution plating. *Tetrahymena* counts and live/dead staining (Thermo LIVE/DEAD™ Baclight™) for *Acanthamoeba* are performed at 1- and 24-hours post infection.
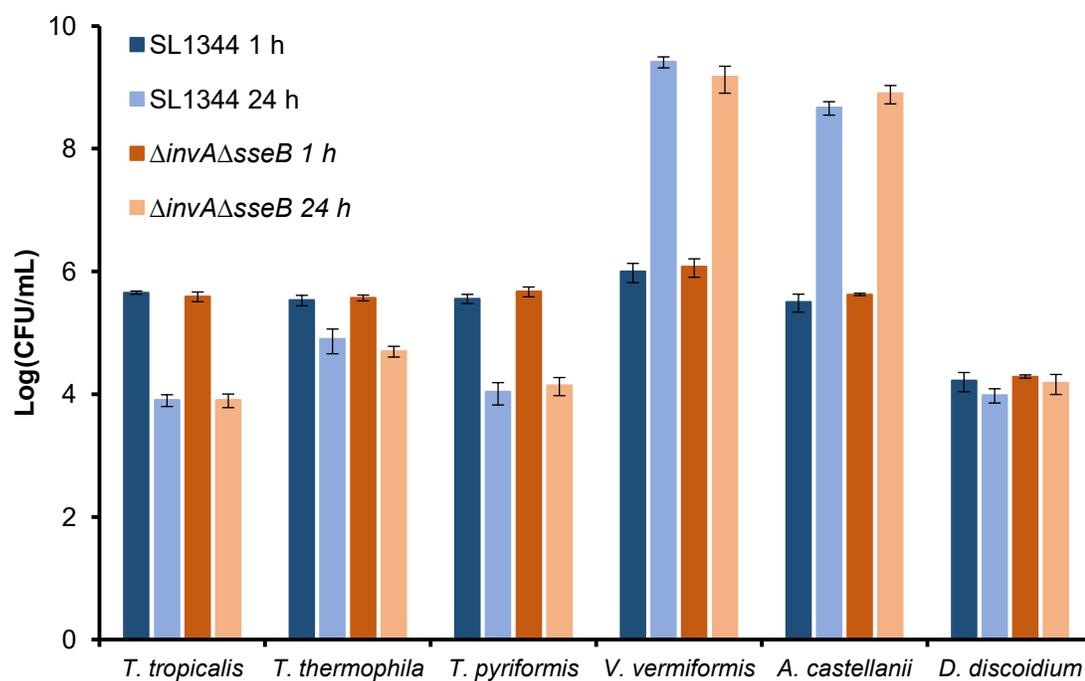
Figure 4.3: *Salmonella* **survival in protozoan grazing assay.**
Bacterial enumeration (log(CFU/mL)) from protozoan grazing assays involving ciliates (*Tetrahymena tropicalis*, *T. theromophila,* and *T. pyriformis*) and amoeba *(Vermamoeba vermiformis, Acanthamoeba castellanii,* and *Dictyostelium discoidium*) with *Salmonella* strains SL1344 (blue) and *ΔinvAΔsseB* (orange). *Salmonella* levels quantified from co-culture aliquots at 1- and 24-hours post-inoculation. Values are the means ± standard deviations of three independent experiments.

Furthermore, neither SL1344 nor Δ*invA*Δ*sseB* levels were different at either time point in *Dictyostelium* grazing assays. Therefore, T3SS-1 and T3SS-2 together do not contribute to grazing resistance in the six protozoa species I tested.

Aside from bacterial enumeration, I was interested in quantifying protozoan growth and viability over the course of grazing assays. Previous research has demonstrated that *Tetrahymena* spp. grow during *Salmonella* co-culturing experiments (655). In all three *Tetrahymena* species tested, ciliate counts increased slightly between the 1-hour and 24-hour time points (Figure 4.4A). However, these increases were unaffected by defects in T3SS-1 and T3SS-2. Next, I assessed amoeba viability in co-culturing experiments as prior research has demonstrated that *Acanthamoeba* spp. lose viability when co-cultured with *Salmonella* (182). I used the fluorescent dyes SYTO-9 and PI to stain viable and dead *A. castellanii* and measured differences at the 1-hour and 24-hour time points (Figure 4.4B). The proportion of PI stained amoeba (dead) increased between the two time points, while the percentage of amoeba exhibiting only SYTO-9 associated fluorescence decreased. However, the increase in PI stained amoeba was independent of T3SS-1 and T3SS-2 and was similar to the increase observed in the uninoculated control. Therefore, T3SS-1 and T3SS-2 together do not affect *Tetrahymena* growth or *A. castellanii* viability during *Salmonella* grazing assays.

4.2.2. *Salmonella* Virulence in *Acanthamoeba* spp. Gentamicin Protection Assay

Considering the protozoan grazing assays could not distinguish between SL1344 and Δ*invA*Δ*sseB*, I adopted experimental conditions akin to those used for *Salmonella* infections in mammalian cells (52). Unlike *Tetrahymena* spp. and *Dictyostelium*,

A.

B.
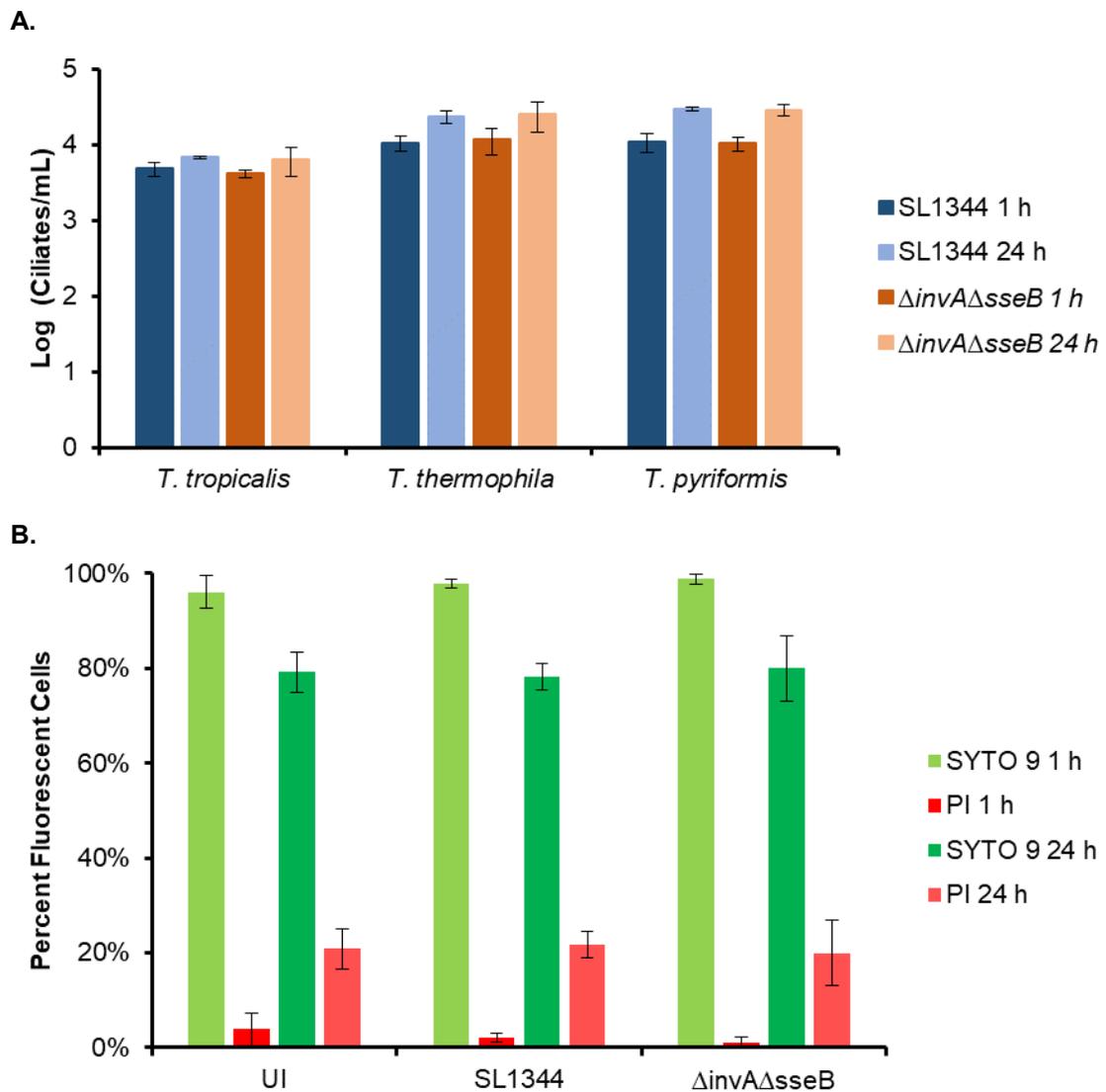
Figure 4.4: Ciliate replication and amoeba viability in protozoan grazing assay.
(A) *Tetrahymena* counts from grazing assays during incubation with SL1344 or
Δ*invA*Δ*sseB* recorded at 1 (blue) and 24 hours (orange). Values are the means ± standard
deviations of three independent experiments. (C) Live/dead quantification of *A.
castellanii* using SYTO 9 (alive, green) and propidium iodide (PI, dead, red) staining.
Quantification of fluorescent amoeba recorded following incubation with SL1344 or
Δ*invA*Δ*sseB* for 1 or 24 hours. Values are the means ± standard deviations of three
independent experiments.

Acanthamoeba spp. adhere to inert surfaces similar to mammalian macrophages. Consequently, the adherent amoebae are conducive to internalization assays that involve numerous wash steps, such as the gentamicin protection assay (GPA). Several studies have used gentamicin protection assays in *Acanthamoeba* spp. to demonstrate the importance of T3SS-2 to intracellular survival of *Salmonella* (176,181,182). Using these studies as guidelines, I performed GPAs with two time points in both *Acanthamoeba rhysodes* and *Acanthamoeba polyphaga* to evaluate internalization (0 hours) and intracellular replication (24 hours) phenotypes of SL1344 and Δ*invA*Δ*sseB* (Figure 4.5).

GPAs performed in both *A. rhysodes* and *A. polyphaga* displayed decreases in SL1344 and Δ*invA*Δ*sseB* between the 0-hour and 24-hour time point (Figure 4.6). While the reduction in *Salmonella* is similar between both strains, the internalization of Δ*invA*Δ*sseB* is a half-log lower than SL1344 in both *Acanthamoeba* species. I tested two additional *Salmonella* strains, S5 (*Salmonella* ser. Enteritidis) and S37 (*Salmonella* ser. Falkensee), from the Syst-OMICS isolate consortium that display high and low virulence phenotypes in *in vitro* mammalian cell culture models, respectively (656). While S5 displayed a higher internalization level relative to SL1344, S37 was recovered in much lower amounts in both *Acanthamoeba* species (Figure 4.6). Recovered CFUs for both S5 and S37 also decreased between the 0-hour and 24-hour time points, but the reductions were similar in magnitude. Altogether, GPAs performed in *A. rhysodes* and *A. polyphaga* can distinguish between high- and low-virulence *Salmonella* strains via enumeration of internalized bacteria. This amoeba infection assay serves as the basis for my *Salmonella* virulence screening model.
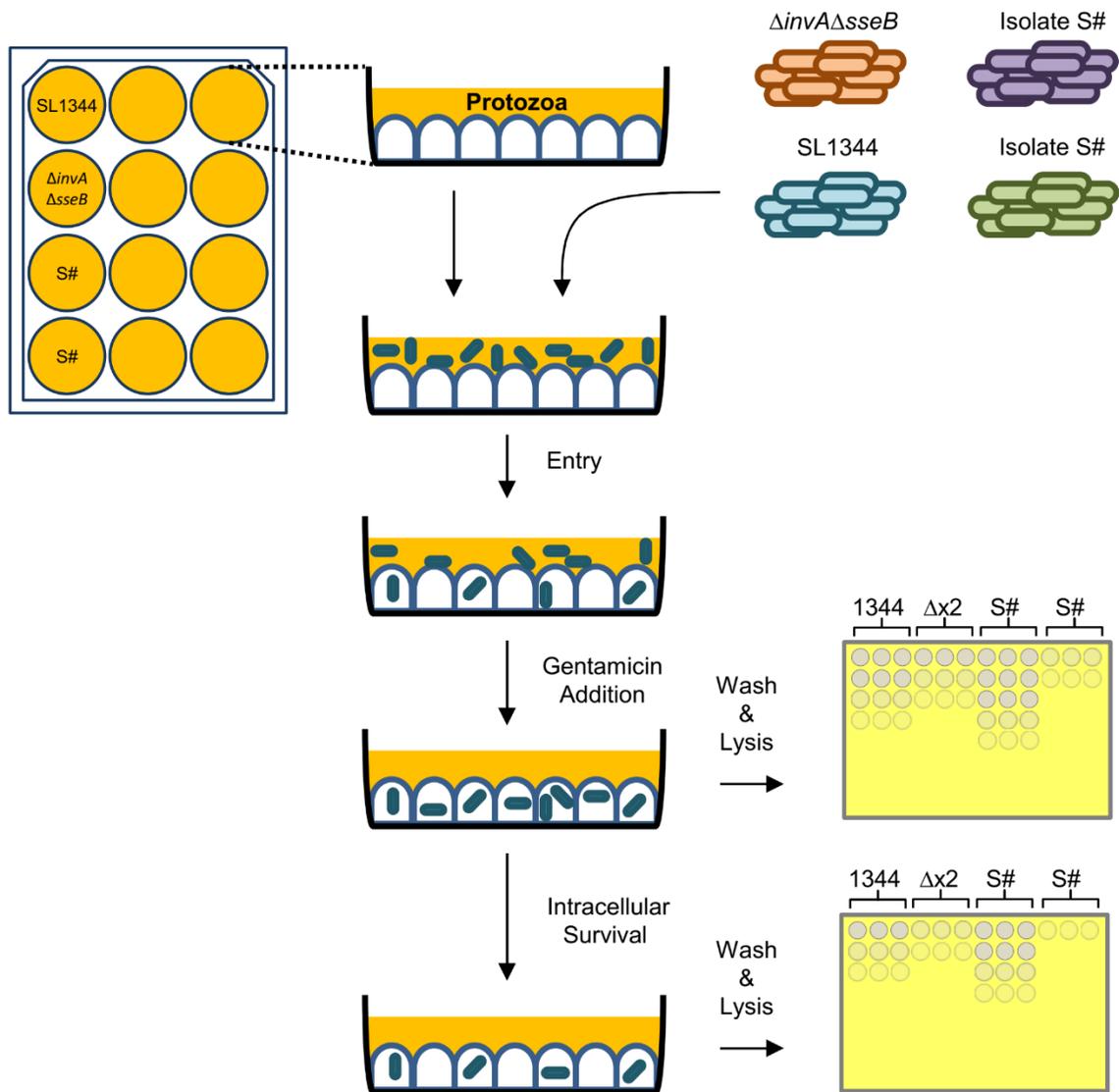
Figure 4.5: Evaluation of *Salmonella* internalization and intracellular replication using an *Acanthamoeba* spp. gentamicin protection assay.
A gentamicin protection assay is used to evaluate *Salmonella* entry and intracellular survival in *Acanthamoeba*. Both SL1344 and Δ*invA*Δ*sseB* are used as reference strains. Wells seeded with *Acanthamoeba* in modified peptone-yeast extract-glucose (mPYG) are inoculated with control strains and *Salmonella* isolates at an MOI of 10 and incubated at 30°C. Medium is replaced for each well with mPYG containing gentamicin at 50µg/mL following 1 hour incubation. Time zero is established after 0.5-hour incubation in the gentamicin containing media. Amoeba are washed with Page's amoeba saline (PAG) and are either lysed with 0.5% deoxycholate for dilution plating or incubated in mPYG with 10µg/mL gentamicin for 24 hours followed by lysis.
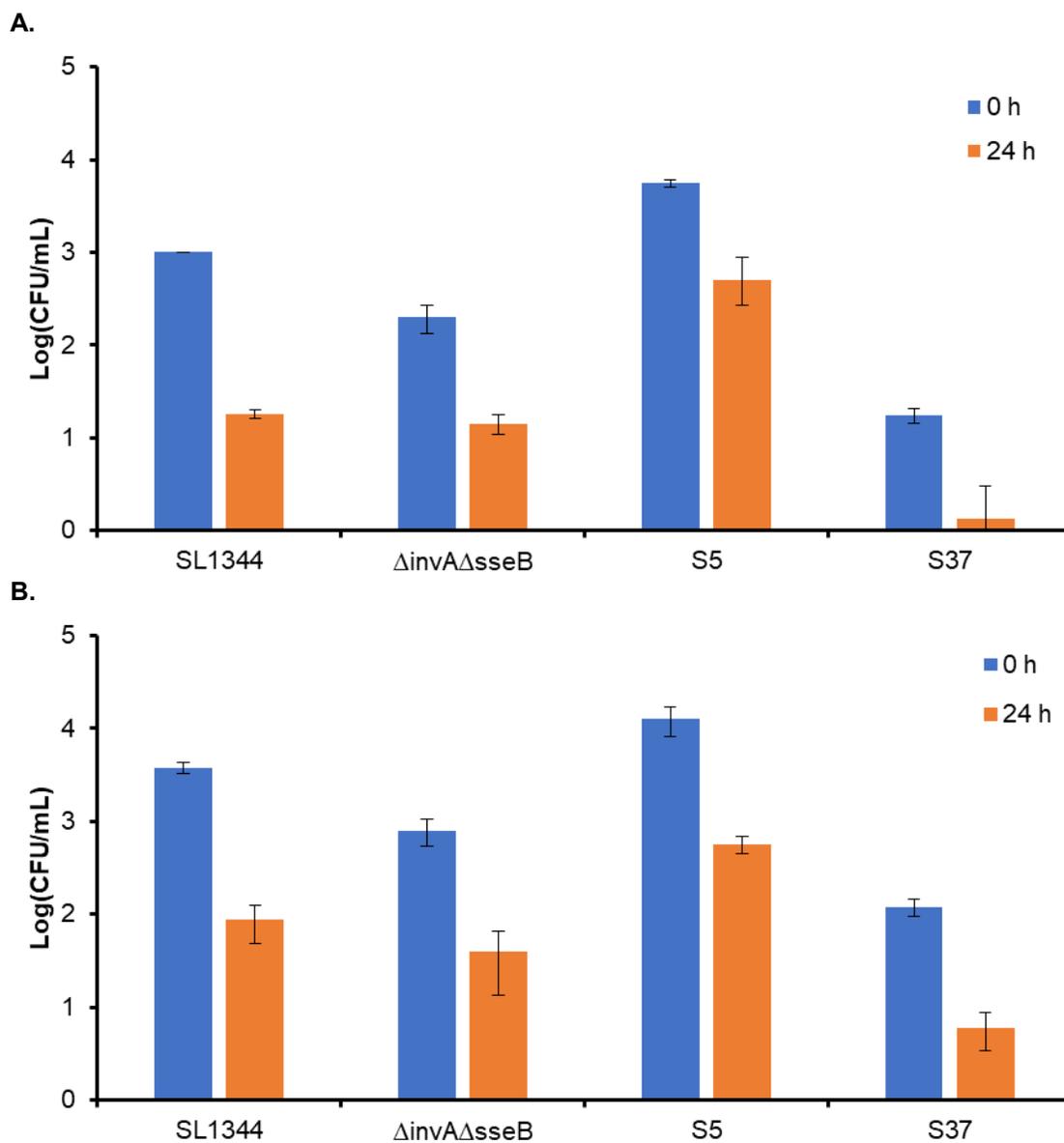
Figure 4.6: *Salmonella* **entry and intracellular survival in** *Acanthamoeba* **gentamicin protection assay.**
Enumeration (log (CFU/mL)) of SL1344 and ΔinvAΔsseB recorded at 0 (entry, blue) and 24 hours (intracellular survival, orange) from gentamicin protection assays in *A. rhysodes* (A) and *A. polyphaga* (B). Values are the means ± standard deviations of three independent experiments.

4.2.3.   Screening of *Salmonella* Isolates through the *A. rhysodes* Infection Model

The GPAs performed in *A. polyphaga* and *A. rhysodes* were conducted to identify a suitable protozoan model that will distinguish between high- and low-virulence *Salmonella* strains. Infections in both amoeba species demonstrated notable differences in internalization and intracellular replication levels between SL1344, Δ*invA*Δ*sseB*, S5, and S37. I opted to only use *Salmonella* internalization as a measure of virulence to enhance efficiency of the mid-throughput screening model. Additionally, I chose *A. rhysodes* as the screening host as several studies have used the *Acanthamoeba* species to evaluate virulence in *Salmonella* (176,182).

I evaluated internalization of 107 clinical and environmental isolates from the *Salmonella* Syst-OMICS isolate consortium using the *A. rhysodes* screening model (Figure 4.7). The screen produced a range of internalization phenotypes among the tested isolates with SL1344 and Δ*invA*Δ*sseB* serving as reference strains. Nearly half of the isolates I screened displayed internalization levels in between SL1344 and Δ*invA*Δ*sseB*. Again, the entry levels of isolates S5 and S37 highlight the range of the *A. rhysodes* GPA as S5 exhibited the highest degree of internalization, whereas S37 ranked near the bottom of screened isolates. Relative to S37, five isolates (S219, S26, S229, S189, and S49) displayed lower recovery yields indicating poor internalization rates. Additionally, several isolates internalized into *A. rhysodes* to a greater degree than SL1344, suggesting an increased capacity to enter amoeba. While the internalization screen identified *Salmonella* isolates with high and low levels of entry, it was necessary to confirm these observations in different *Acanthamoeba* species.
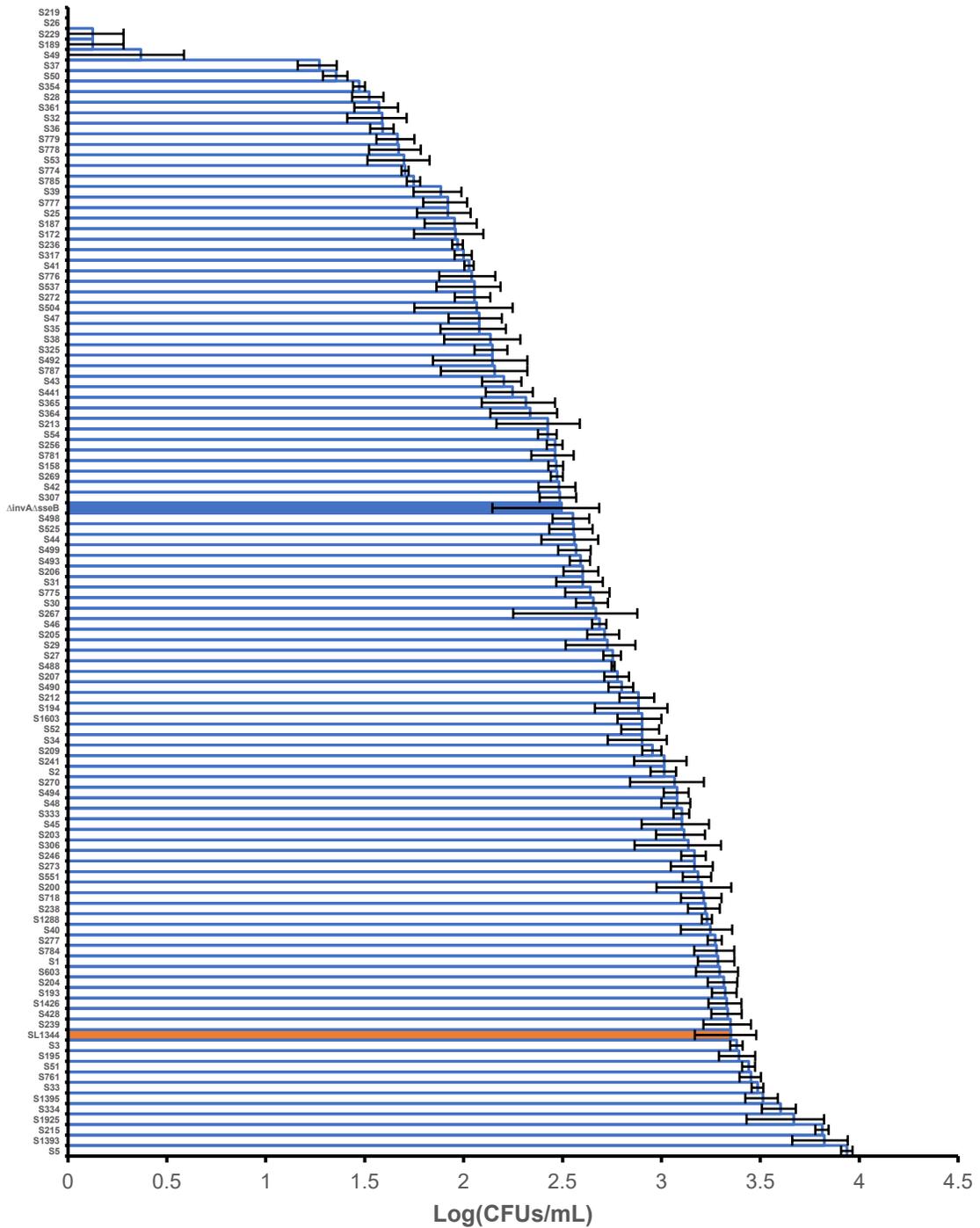
Figure 4.7: *Salmonella* Syst-OMICS isolate screening through *A. rhysodes* infection model.

Enumeration (log (CFU/mL)) of *Salmonella* isolates from gentamicin protection assays performed in *A. rhysodes*. Reference strains SL1344 and Δ*invA*Δ*sseB* are coloured solid orange and blue, respectively. Values are the means ± standard deviations of three independent experiments.

I next validated high- and low-internalization phenotypes observed in screened isolates through re-testing the strains in *A. castellanii* and *A. polyphaga* gentamicin protection assays (Figure 4.8). Isolates S26, S49, S50, and S189 all displayed low internalization phenotypes in the *A. rhysodes* screening, whereas isolates S33, S51, S195, and S215 exhibited high internalization phenotypes. In *A. castellanii* and *A. polyphaga*, S26, S49, S50, and S189 exhibited low degrees of internalization, although S50 recovery levels were noticeably higher in both *Acanthamoeba* species relative to the other low entry isolates. Regarding high internalization phenotypes, S33, S51, S195, and S215 all displayed internalization levels higher or similar to SL1344. Isolate internalization levels were comparable between *A. castellanii* and *A. polyphaga*, although, recovery yields were on average lower in *A. castellanii*, especially for isolates S26, S49, and S189. Collectively, these results confirm the phenotypes identified in the internalization screen and provide support for *A. rhysodes* as a model to evaluate *Salmonella* entry.

4.3. Discussion

Several studies have used protozoa to screen transposon mutant libraries to characterize genetic contributions to predation resistance and intracellular survival (657–661). Regarding *Salmonella¸* multiple studies have evaluated the impact of specific virulence factors on survival in protozoa (162,169,181), but no protozoan-based screenings to characterize virulence in *Salmonella* mutant libraries or isolate collections have been conducted. In this section, I evaluated the capacity of various protozoan assays to distinguish between high- and low-virulence *Salmonella* strains and subsequently screened isolates through the most discriminatory model to characterize *Salmonella*
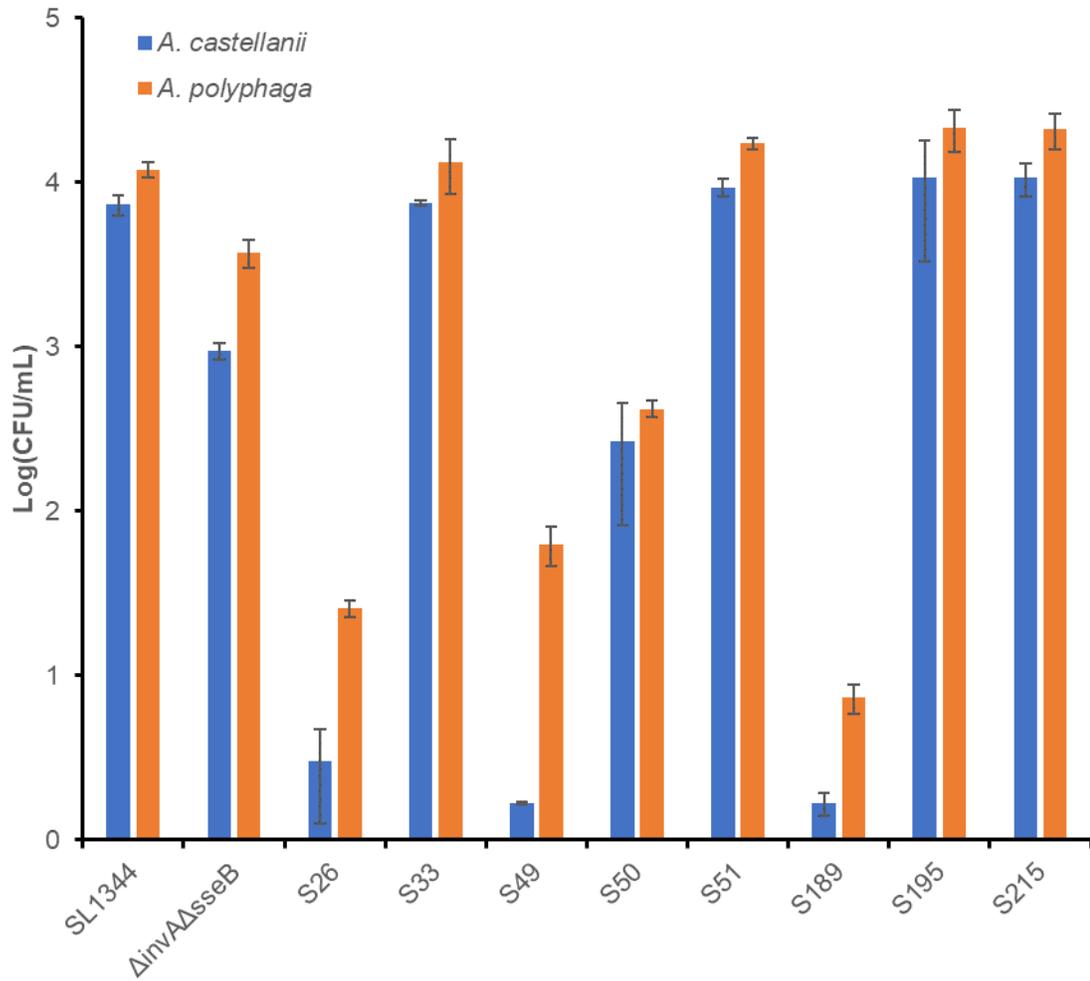
Figure 4.8: Validation of internalization phenotypes in *Acanthamoeba* spp.
Enumeration (log (CFU/mL)) of isolates with high (S33, S51, S195 and S215) and low
(S26, S49, S50 and S189) internalization phenotypes from gentamicin protection assays
performed in *A. castellanii* (blue) and *A. polyphaga* (orange). Values are the means ±
standard deviations of three independent experiments.

virulence. This study was part of a larger project to model *Salmonella* virulence through combining WGS analysis with virulence profiling of *Salmonella* Syst-OMICs isolates. While I screened over one hundred *Salmonella* strains through the *A. rhysodes* screening assay, collaborating research groups characterized the same isolates through mammalian infection models. The results from these screening assays were consolidated into virulence profiles for each isolate to be analyzed against WGS data for predictive modelling development.

To maintain a standard of consistency, all screening models used to screen *Salmonella* Syst-OMICS isolates were first required to discriminate between the laboratory *Salmonella* ser. Typhimurium strain SL1344 and the double mutant derivative Δ*invA*Δ*sseB*. InvA and SseB are integral to the proper functioning of T3SS-1 and T3SS-2, respectively, which are known contributors to *Salmonella* pathogenesis in mammalian infection models (191,259). The contributions of SPI-1 and SPI-2 are less defined in protozoan infections. SPI-2 associated genes are upregulated inside several different protozoa and contribute to intracellular survival of internalized *Salmonella* (162,169,181,182). Conversely, the contributions of SPI-1 to predation resistance are inconsistent across protozoa as defects in T3SS-1 reduce *Salmonella* levels in *Dictyostelium* (169), but do not impact survival in *Acanthamoeba* (176,181). The collective contributions of T3SS-1 and T3SS-2 secretion to predation resistance are currently unknown as no study has evaluated a *Salmonella* mutant dysfunctional for both T3SSs in a protozoan infection model. To identify a screening model that will distinguish between SL1344 and Δ*invA*Δ*sseB*, I evaluated the discriminatory power of three ciliate species, five amoebae species, and two infection assays.

In the environment, protozoa prey on bacteria as a source of nutrients (646). Protozoan grazing assays simulate this predation through co-culturing bacteria together with protozoa in non-nutrient buffer to promote starvation and phagocytosis. Typically, bacterial levels are enumerated from aliquots directly sampled form the co-culture at multiple time points. These measurements primarily provide insight on changes in extracellular bacteria levels in response to protozoan predation. I performed grazing assays in three different *Tetrahymena* species and three different amoebae to evaluate differences in their predatory capacity for SL1344 and $\Delta invA\Delta sseB$. Specifically, I examined *Salmonella* levels and protozoa fitness for changes linked to T3SS-1 and T3SS-2 activity.

In all three *Tetrahymena* species tested, *Salmonella* levels decreased during co-incubation (Figure 4.3), which corresponds with reports investigating intracellular *Salmonella* viability within ciliate food vacuoles (162). However, this reduction in *Salmonella* levels recorded is independent of T3SS-1 and T3SS-2 activity as both SL1344 and $\Delta invA\Delta sseB$ levels decreased by similar amounts. I also examined *Tetrahymena* growth in response to *Salmonella* co-incubation as ciliate levels typically expand during *Salmonella* co-culture experiments (655). Ciliate counts increased for all three *Tetrahymena* species during grazing assays, but the increase was independent of both T3SS-1 and T3SS-2 function (Figure 4.4A). Considering that ciliate predation was indistinguishable between SL1344 and $\Delta invA\Delta sseB$, I determined that the *Tetrahymena* spp. are not suitable as a virulence screening model for the *Salmonella* Syst-OMICS isolate consortium.

The three amoebae examined in grazing assays were similarly unable to distinguish between SL1344 and Δ*invA*Δ*sseB* (Figure 4.3). In *V. vermiformis* and *A. castellanii*, extracellular *Salmonella* levels increased during co-incubation experiments validating previous findings that extracellular *Salmonella* expand in non-nutrient buffer in the presence of amoeba (662). This increase in *Salmonella* levels was independent of T3SS-1 and T3SS-2 activity as SL1344 and Δ*invA*Δ*sseB* grew by similar amounts. I also examined *A. castellanii* viability during grazing assays (Figure 4.4B), as amoeba exhibit apoptosis-like death in response to high *Salmonella* levels (182). While amoeba viability decreased over time, the differences between SL1344, Δ*invA*Δ*sseB*, and the uninoculated control were negligible, suggesting starvation may be responsible for the increase in amoeba death. In *Dictyostelium* grazing assays, *Salmonella* levels slightly decreased during amoeba co-incubation and was independent of T3SS-1 and T3SS-2 activity. These findings contradict previous reports on *Salmonella-Dictyostelium* experiments demonstrating an expansion of *Salmonella* in a T3SS-1 and T3SS-2 dependent manner (169). However, these experiments were performed in competition assays, which are not conducive to efficient isolate screening. Given that the predation resistance phenotypes were indistinguishable between SL1344 and Δ*invA*Δ*sseB,* I determined that the protozoan grazing assay was not suitable as a screening method of *Salmonella* virulence.

While the protozoan grazing assays I conducted were unaffected by T3SS-1 and T3SS-2 activity, previous grazing experiments have characterized other *Salmonella* virulence factors as contributors to predation resistance. Grazing assays performed in *Tetrahymena* have demonstrated the importance of the arginine decarboxylase system in facilitating *Salmonella* acid tolerance and subsequently maintaining bacterial viability

within ciliate food vacuoles and fecal pellets (162). In *Dictyostelium*, grazing assays establish the importance of polyphosphate biosynthesis and the twin-arginine translocation (Tat) system to intracellular survival (170,171), both of which promote intracellular replication in macrophages as well (663,664). Grazing assays in *Tetrahymena* and *Acanthamoeba* also provide insight into *Salmonella*-related exploitation of protozoan cellular processes for protection from environmental stressors. In *Tetrahymena*, viable *Salmonella* secreted in fecal pellets during grazing experiments possess enhanced resistance to bactericidal concentration of chlorine and bleach (161,665). Furthermore, *Salmonella* remain viable for up to three weeks inside *Acanthamoeba* cysts that are formed in response to environmental stressors during long-term grazing assays (666). Although protozoan grazing assays can characterize *Salmonella* predation resistance and environmental persistence strategies, their discriminatory capacity do not align with the mammalian models involved in screening the *Salmonella* Syst-OMICS isolate consortium.

The second screening assay I evaluated regarding *Salmonella* virulence in protozoa was the GPA. GPAs require adherent cell lines to quantify internalization and intracellular replication of intracellular bacteria. Considering *Tetrahymena* and *Dictyostelium* are both cultured in suspension, I elected to use *Acanthamoeba* spp. as the adherent host for protozoan GPAs. I performed GPAs in *A. polyphaga* and *A. rhysodes* as previous studies have conducted GPAs in each species (181,182). These studies also demonstrated that SPI-2 is upregulated inside amoeba and is required for intracellular survival of *Salmonella*. The GPAs I performed in both *Acanthamoeba* species reflected these reports as I recovered a lower amount of intracellular Δ*invA*Δ*sseB* levels relative to

SL1344 (Figure 4.6). Both *Acanthamoeba* species were also able to characterize *Salmonella* isolates displaying greater internalization, S5 (*Salmonella* ser. Enteritidis), and reduced internalization, S37 (*Salmonella* ser. Falkensee), relative to SL1344. Although, intracellular levels for all four *Salmonella* strains decreased between 0 and 24 hours, the extent of reduction was similar for each strain. Therefore, I opted to remove the 24-hour time point to enhance efficiency in screening the *Salmonella* Syst-OMICS isolate consortium.

The *Salmonella* isolate screening I performed used *A. rhysodes* as it exhibits a greater capacity for *Salmonella* phagocytosis compared to other *Acanthamoeba* species (176). This screening successfully evaluated the internalization of 107 *Salmonella* isolates displaying varying degrees of entry into *A. rhysodes* (Figure 4.7). Interestingly, four isolates (S26, S49, S189, S219, and S229) appear to barely internalize at all, while three isolates (S5, S1393, and S215) internalized to a greater degree relative to SL1344. I validated these observations through re-examining internalization phenotypes of select isolates from the *A. rhysodes* screen in *A. polyphaga* and *A. castellanii* GPAs (Figure 4.8). Although, internalization levels between *A. polyphaga* and *A. castellanii* for strains recovered in low amounts were variable, all re-examined isolates exhibited similar entry phenotypes. Altogether, these findings support *Acanthamoeba* GPAs as an effective model to screen *Salmonella* internalization in protozoa.

While the *Salmonella* Syst-OMICS screening in *A. rhysodes* produced a range of internalization phenotypes, the exact genetic contributors responsible for the variance in isolate entry remain to be determined. Other intracellular pathogens such as *Legionella pneumophila* and *Mycobacterium* spp. rely on conserved mechanisms in intramacrophage

survival to grow within amoeba (154,667). By comparison, it is likely that many *Salmonella* virulence factors that influence internalization and intramacrophage survival may also apply to amoeba. The most well characterized contributors to internalization and intracellular replication in mammalian cells, SPI-1 and SPI-2, respectively, appear to be important for amoeba internalization as indicated by the reduction in internalization with Δ*invA*Δ*sseB*. However, prior reports demonstrate that SPI-1 is largely dispensable for internalization into *Acanthamoeba*, whereas SPI-2 contributes to both entry and intracellular survival (176,181). Although the contributions of SPI-2 to intracellular survival are well documented (653,654), it is apparent that SPI-2 is not solely responsible for *Salmonella* entry into amoeba as Δ*invA*Δ*sseB* is capable of internalizing into *Acanthamoeba* spp.

Aside from SPI-1 and SPI-2, several other SPIs associated with intracellular survival are upregulated inside *Acanthamoeba* during *Salmonella* infection (182). Considering the heterogeneity of SPI distribution among *Salmonella* serovars (188), their presence or absence may contribute to internalization and intracellular survival in amoeba. For example, SPI-3 and SPI-11 promote intracellular survival within macrophages (249,310,341,342), are upregulated inside *A. rhysodes* (182), and are differentially distributed amongst *Salmonella* serovars (188). Other mobile elements encoding virulence factors, such as prophage, may also contribute to *Salmonella* invasion and intracellular survival in amoeba. In particular, the prophage *Gifsy-2* encodes a superoxide dismutase, *sodC1*, that contributes to survival in macrophages (668) and may also potentially protect against superoxide production in amoeba (669). *Salmonella* internalization into amoeba is also influenced by O-antigen modifications (670), which

are partly governed by the glucosyltransferase clusters *rfb* and *gtr*, found in prophage ε^{34} and P22 (417). Further investigation into these virulence determinants are required to establish their contributions to *Salmonella* internalization and intracellular survival in amoeba.

The work presented in this section on the characterization of *Salmonella* virulence through protozoan-based assays is a part of a much larger effort to model *Salmonella* pathogenicity. While the protozoan grazing assays I evaluated displayed inadequate discriminatory capacity in measuring *Salmonella* virulence, *Acanthamoeba* GPAs emerged as a suitable model to characterize isolates from the Syst-OMICs collection. My work screening the *Salmonella* Syst-OMICS isolate consortium through the *A. rhysodes* infection model represents the first instance of protozoan-based virulence characterization of clinical and environmental *Salmonella* isolates. This screening correlated well with mouse infections and intramacrophage survival screenings that were also used to characterize the virulence of isolates from the *Salmonella* Syst-OMICS consortium (656). Combined with the discriminatory power displayed in the internalization screening, these findings highlight the utility of *Acanthamoeba* as a *Salmonella* infection model.

Chapter 5: Degradation of Protein Kinase N1 by SspH1 during *Salmonella* infection

5.1. Introduction

As an intracellular pathogen, *Salmonella* is equipped with a wide range of strategies to facilitate survival within a host. One tactic *Salmonella* uses to establish its intracellular niche is through the secretion of protein effectors into the host cytosol to modulate a variety of cellular processes. Many of these effectors are regulated in parallel with SPI-1 and SPI-2 and are highly conserved across *S. enterica* serovars (671). While the contributions of some effectors to *Salmonella* internalization and intracellular replication are well defined, the role of other secreted proteins during infection remain unclear. This gap in effector characterization is exemplified in the *Salmonella* secreted enzyme SspH1, which is a focus of my thesis.

Miao *et al.* first identified SspH1 and its functionally homologous relative SspH2 as LRR containing proteins in the *Salmonella* chromosome (412). Unlike *sspH2* which is expressed from the highly conserved SPI-12 (344), *sspH1* is encoded in the *Gifsy-3* prophage element and is primarily found in non-*enterica Salmonella* subspecies (399). Early experiments with SspH1 revealed its contributions to infection as a double mutant deleted for both *sspH1* and *sspH2* is attenuated in a diarrheal calf model (412). Conversely, a *sspH1* isogenic mutant produces similar calf intestine pathology compared to wild type (672). More recently, mouse experiments demonstrated that SspH1 contributes to persistence during systemic infection (673). Although these observations implicate SspH1 in enhancing *Salmonella* infection, a disconnect remains between the ascribed activity of SspH1 and its clinical contributions.

During infection, SspH1 is secreted by both T3SS-1 and T3SS-2, after which it localizes to the host nucleus where it has been reported to suppress NF-κB activity and pro-inflammatory cytokine expression (235). In addition to NF-κB suppression, SspH1 also manipulates the host ubiquitin system as it is part of the NEL-type E3 family, displaying ubiquitin ligase activity analogous to the IpaHs found in *S. flexneri* (512). PKN1 was identified as a protein that interacted with SspH1 using a yeast 2-hybrid screen and subsequent biochemical analysis (542). Subsequent studies demonstrated that SspH1 could directly ubiquitinate PKN1 *in vitro* using purified proteins (512). Evidence that SspH1 facilitates ubiquitination of PKN1 leading to its degradation has been provided using transfection experiments (543). It remains to be determined how SspH1-dependent ubiquitination of PKN1 influences host process during infection.

PKN1 (also named PKNα and PRK1) is one of three protein kinase C-related kinase isoforms (674). As a serine/threonine kinase, PKN1 is associated with phosphorylating over 20 proteins (675) and is involved in regulating several cellular processes including cell migration (676,677), receptor trafficking (678), vesicle trafficking (679–681), hormone receptors (682), p38 signaling (683,684), and inflammasome activation (527,685). This kinase activity is encoded in the C-terminal region of PKN1, while the N-terminus is home to homology region 1 (HR1) a, b, and c (Figure 5.1). The Rho-GTPases RhoA, RhoB, and RhoC bind to the HR1 of PKN1 and regulate its activity via recruitment of phosphoinositide-dependent kinase 1 (PKD1), which phosphorylates the kinase activation loop (686–688). PKN1 also contains an auto-inhibitory region within the C2-like domain (689) and several caspase-3 cleavage sites that can produce a
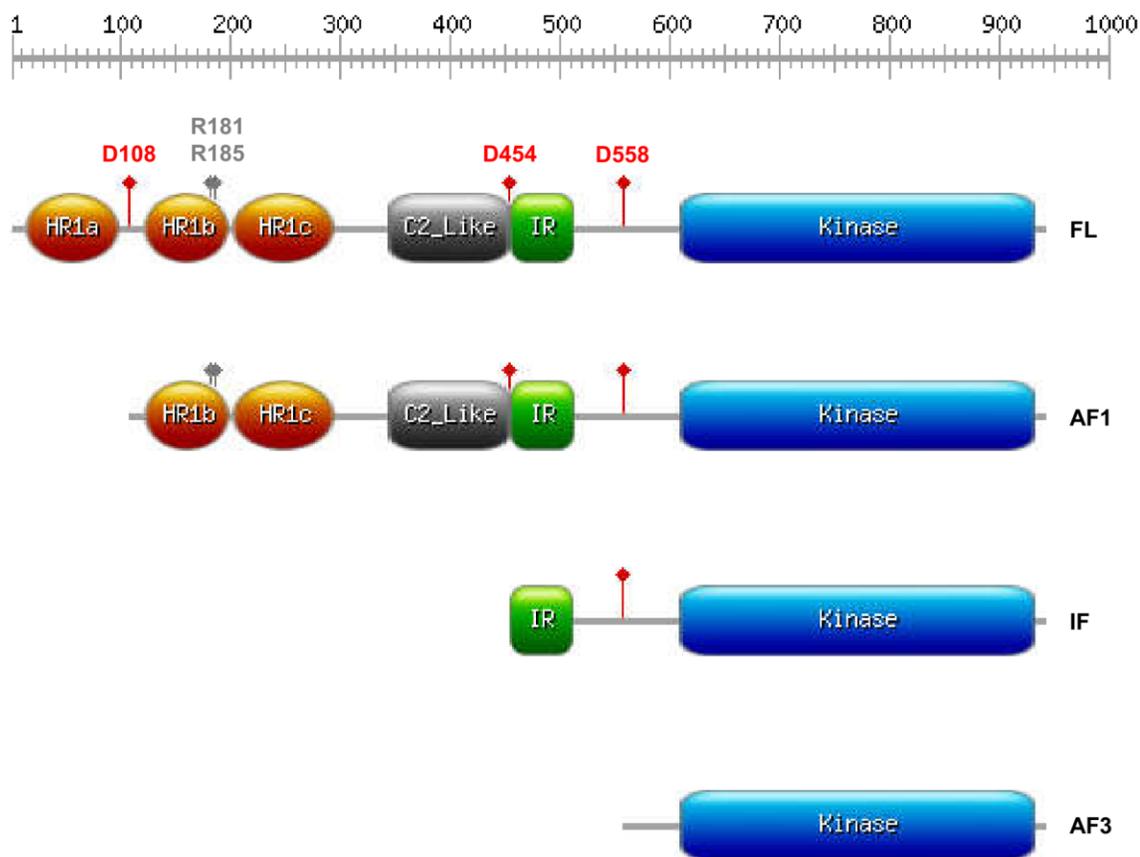
Figure 5.1: Protein map of Protein Kinase N1
PKN1 is composed of a set of anti-coil coiled domains referred to as homology region 1 (HR1) a, b, and c. HR1a and HR1b primarily serve as the binding sites for Rho-GTPases, which regulate PKN1 activity. The C2-like domain is weakly homologous to the C2 domain found in protein kinase C (PKC) and contains an auto-inhibitory region (IR) at its C-terminus that regulates PKN1 kinase activity. The PKN1 kinase domain is located at the C-terminal region and contains an activation loop, which is phosphorylated at T774 by phosphoinositide-dependent kinase 1 (PDK1) for activation. PKN1 contains three caspase-3 cleavage sites that are cleaved in response to caspase-3 activation. This cleavage produces three distinct fragments from full-length (FL) PKN1 (~120 kDa). Apoptotic fragment (AF)1 (105 kDa), AF2 (not shown), and AF3 (55 kDa) are observed during caspase-3 associated cell death. AF2 (90 kDa) is formed from an unidentified cleavage site. An additional fragment 70 kDa, referred to as an intermediate to AF3 (IF), is only observed *in vitro*. PKN1-AF3 displays constitutively active kinase activity due to the loss of the IR. SspH1 interacts with R181 and R185 of PKN1 and ubiquitinates lysine residues, of which there are 41 the PKN1 amino acid sequence. Figure generated using ExPASy PROSITE MyDomains.

constitutively active truncated form of PKN1 upon caspase-3 activation (690,691). Consequently, PKN1-associated signal transduction activity is high during caspase-3 related cell death (690).

PKN1 activity is involved in suppressing activation of the serine/threonine kinase Akt, a master regulator of anti-apoptotic signaling (692). Specifically, *Pkn1* knockout mice display increased basal Akt activation in mice and in cerebellar granule cells (693,694). As an intracellular pathogen, *Salmonella* stimulates Akt signaling to promote host survival and intracellular replication (243). Whether SspH1 influences Akt signaling during *Salmonella* infection via its interaction with PKN1 is unknown. I hypothesize that the enhancement of *Salmonella* infection related to SspH1 is likely linked to cellular processes that PKN1 regulates. The objective of this work was to characterize the contributions of SspH1 to Akt signaling in relation to PKN1 activity during *Salmonella* infection.

5.2. Results

5.2.1. PKN1 Expression and Processing

PKN1 is differentially expressed across mammalian cell types with low levels reported in HeLa and HT29 cells (676), which are commonly used cell lines for *in vitro* *Salmonella* infection. Immortalized monocytes, such as U937s, exhibit higher mRNA levels of PKN1 by comparison (695,696) and are also often used to study *Salmonella* interactions with phagocytes (52,697,698). I evaluated PKN1 levels in cell lines used to model *Salmonella* infection *in vitro* to compare protein expression between cell types (Figure 5.2A). Immunoblots on HeLa, HEK293A, HT29, and U937 cell lines
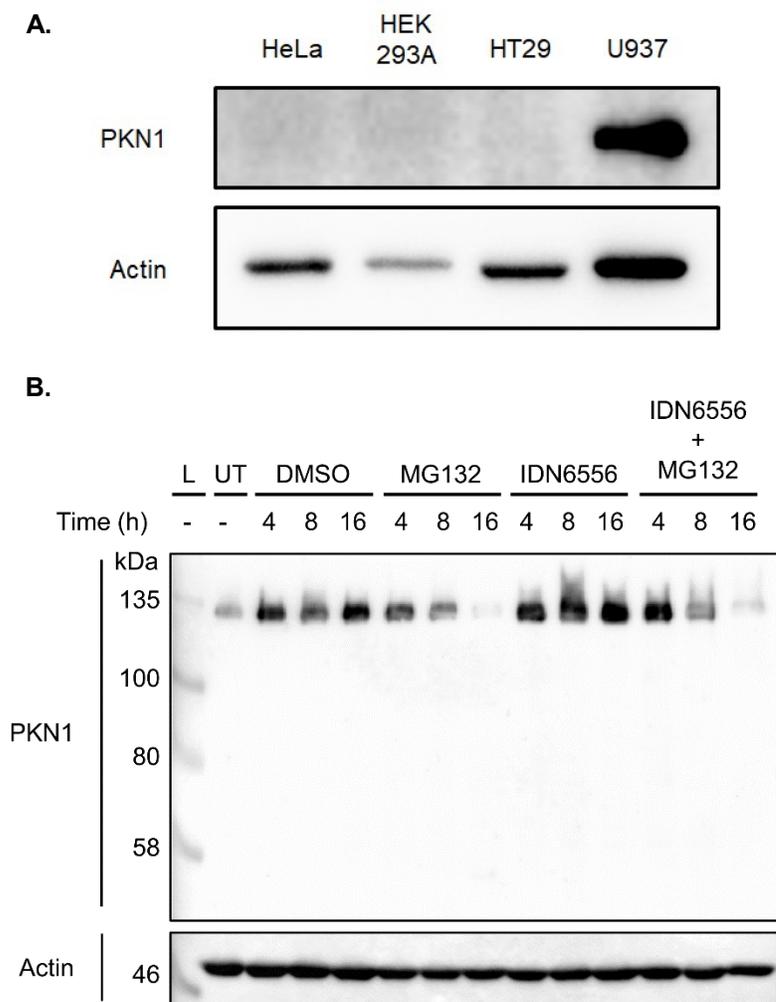
Figure 5.2: PKN1 distribution and cleavage.
(A) PKN1 protein levels in various cell lines. Immunoblot analysis using anti-PKN1 on HeLa cells (cervix epithelial), HEK293A (kidney epithelial), HT29 (colon epithelial), and U937 (monocyte) lysates. Actin immunoblot serves as loading control. Blot is representative of three independent experiments. **(B)** U937 PKN1 protein levels in response to proteasome and pan-caspase inhibition. Immunoblot analysis using anti-PKN1 on U937 cells treated with DMSO (10μM), MG132 (10μM), IDN-6556 (10μM), or both MG132 and IDN-6556 for 4, 8, or 16 hours. Untreated (UT) U937 lysate included as a negative control. Molecular weight ladder (L) included to identify cleavage fragments of PKN1. Molecular weight scale listed in kiloDaltons (kDa). Actin immunoblot serves as loading control. This blot is representative of three independent experiments.

demonstrated that high levels of PKN1 were present in monocytes, whereas PKN1 was not detected in the epithelial cells. Therefore, I used U937 cells to study PKN1 in *Salmonella* infection as protein levels were present in the monocytes at a detectable level.

To confirm proteasomal degradation of an E3 ubiquitin ligase substrate, such as PKN1, the proteasome inhibitor MG132 is typically used to prevent ubiquitin-related degradation. However, in U937 cells, MG132 treatment stimulates caspase-3 activation and promotes apoptosis (699), which fragments PKN1 through caspase cleavage (690). To prevent MG132-related caspase-3 activation, I treated U937 cells with the pan-caspase inhibitor IDN-6556 (also called emricasan) (Figure 5.2B). Immunoblots on lysates from IDN-6556 treated U937 cells showed greater levels of PKN1 relative to the DMSO control, whereas PKN1 levels were reduced with MG132 treatment. Treatment with both IDN-6556 and MG132 also reduced PKN1 levels, suggesting that MG132 stimulates PKN1 cleavage despite pan-caspase inhibition. Additionally, no cleavage products were detected in U937 lysates treated with MG132 or MG132 and IDN-6556. The absence of cleavage fragments is attributed to the PKN1 antibody as it binds an epitope in the N-terminal region that is lost following capsase-3 activation. These findings indicate that MG132 treatment in U937 cells is incompatible for examining PKN1 degradation. Based on these results, I used IDN-6556 treatment to assess the fate of PKN1 during *Salmonella* infection.

5.2.2. SspH1-Dependent Degradation of PKN1 during *Salmonella* Infection

Numerous studies have confirmed PKN1 as the substrate for SspH1 (512,542–544). However, no research group has proven that SspH1 is responsible for PKN1 degradation during *Salmonella* infection. To investigate PKN1 degradation, I performed

infections in U937 cells with the laboratory *Salmonella* ser. Typhimurium strain 14028, as it encodes *sspH1*, and an isogenic *sspH1* mutant (Δ*sspH1*). Immunoblots of U937 infection lysates showed lower levels of PKN1 in wild type infected cells (14028) beginning at the 3-hour time point relative to Δ*sspH1* infection lysates (Figure 5.3A). I conducted similar infections in IDN-6556 pre-treated U937 cells to assess the impact of caspase activation on full length PKN1 levels (Figure 5.3B). Immunoblots of pre-treated U937 infection lysates displayed a notable difference in PKN1 levels at 3-hours post-inoculation. In DMSO pre-treated U937 cells, PKN1 was still present, migrating as a higher species, in Δ*sspH1* infection lysates, whereas PKN1 levels were faint at 3 hours post wild type inoculation. By comparison, PKN1 detection was markedly greater in IDN-6556 pre-treated U937 cells, particularly at the 3-hour time point. These higher levels of PKN1 in IDN-6556 pretreated cells suggest caspase activation is contributing to the reduction of full length PKN1. Furthermore, the discrepancy in PKN1 levels between wild type and Δ*sspH1* infection lysates with IDN-6556 pre-treatment indicate SspH1 activity is responsible for this difference in detection. To confirm the role of SspH1 in PKN1 degradation during *Salmonella* infection, I generated a complemented strain of Δ*sspH1*. Immunoblots of U937 lysates from infection with the complement Δ*sspH1* strain showed a substantial reduction in PKN1 levels, even more so than wild type infection (Figure 5.3C). Altogether, these results suggest that SspH1, in addition to caspase activation, contributes to PKN1 degradation during *Salmonella* infection.

5.2.3. PKN1, SspH1, and Akt Activation during *Salmonella* Infection

Akt activation is regulated by both *Salmonella* infection and PKN1 activity. In
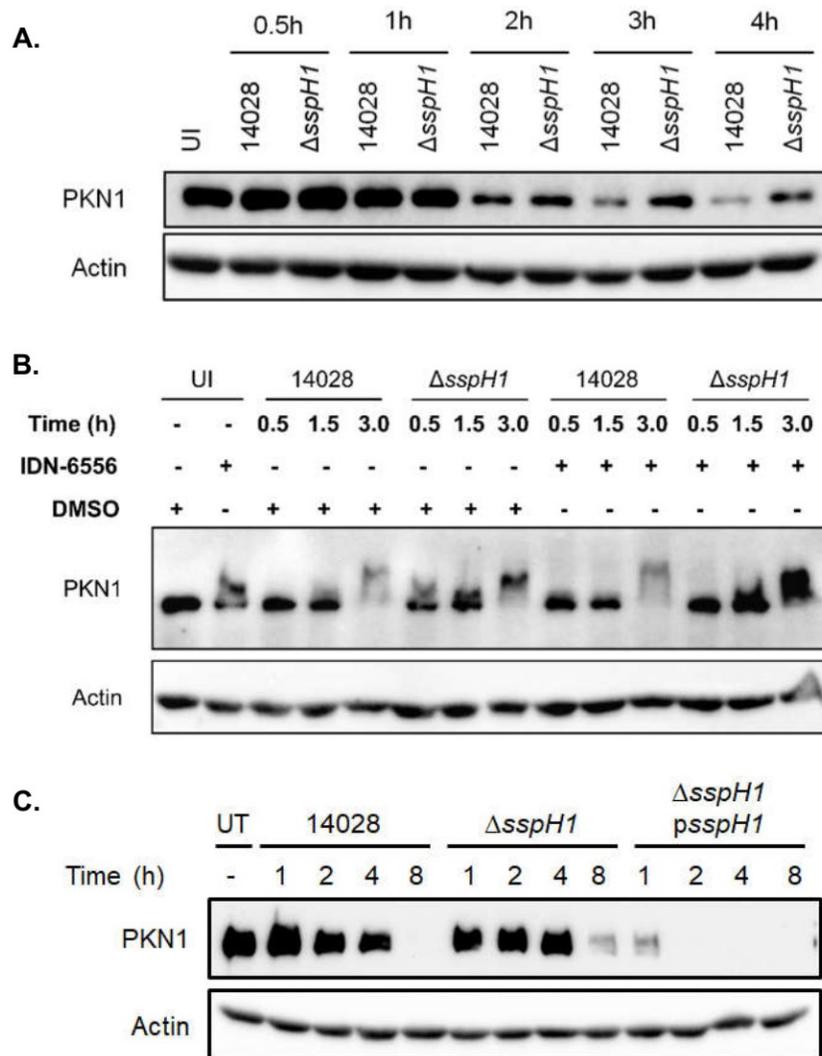
Figure 5.3: Degradation of PKN1 by SspH1 during *Salmonella* infection.
(A) Reduction in PKN1 degradation with *sspH1* knockout. Immunoblot analysis using anti-PKN1 on U937 cells infected with either wild-type (14028) or a *sspH1* knockout mutant (Δ*sspH1*) at a MOI of 30. Lysates are from 1-, 2-, 3-, and 4-hours post inoculation. Uninoculated (UI) lysate included for reference. Actin immunoblots serves as loading control. Blot is representative of three independent experiments. **(B)** Pan-caspase inhibition on PKN1 degradation during *Salmonella* infection. Immunoblot analysis using anti-PKN1 on pre-treated U937, with DMSO (10µM) or IDN-6556 (10µM), infected with either 14028 or Δ*sspH1* at a MOI of 30. Lysates are from 0.5-, 1.5-, and 3-hours post inoculation. Actin immunoblot serves as loading control. Blot is representative of three independent experiments. **(C)** Restoration of PKN1 degradation through *sspH1* complementation. Immunoblot analysis using anti-PKN1 on U937 cells infected with either 14028, Δ*sspH1* with empty vector (pWSK129), or complemented Δ*sspH1* (p*sspH1*) at a MOI of 10. Lysates are from 1-, 2-, 4-, and 8-hours post inoculation. Actin immunoblot serves as loading control. Blot is representative of three independent experiments.

macrophages, *Salmonella* infection stimulates Akt activation, indicated by phosphorylation at serine 473 (S473), to promote intracellular survival (243). Conversely, Akt activity is suppressed by PKN1 under activating conditions and is upregulated in *Pkn1* knockout mice (693). To investigate the impact of PKN1 on Akt activation in macrophages, I generated a PKN1 knockdown (KD) using short hairpin RNA (shRNA) in U937 cells. I first performed serum starvation experiments in PKN1 KD cells to confirm the absence of Akt suppression outside of *Salmonella* infection (Figure 5.4A). Visible increases in phospho-Akt (p-Akt) S473 were apparent when comparing immunoblots for PKN1 KD U937 cells to the non-targeting (Nt) shRNA controls. Specifically, higher p-Akt S473 levels were present at basal condition, during serum starvation, and after replenishing serum in PKN1 KD U937 cells. Total Akt levels also remained consistent between PKN1 KD and Nt shRNA U937 cells indicating differences in Akt phosphorylation are not due to changes in Akt levels. Together, these results suggest that PKN1 suppresses Akt phosphorylation at basal and serum starvation conditions.

I next examined the contributions of PKN1 to Akt activation during *Salmonella* infection. To investigate the impact of SspH1-dependent PKN1 degradation on Akt activation, I assessed p-Akt S473 levels from *Salmonella* infection lysates (Figure 5.4B). Immunoblots of wild type infection lysates displayed an absence of PKN1 at 8- and 16-hours post-inoculation, whereas PKN1 was still present in Δ*ssspH1* infections. This reduction of PKN1 does not appear to affect Akt activation, as p-Akt S473 levels were similar between wild type and Δ*sspH1* infection lysates at the later time points. I also
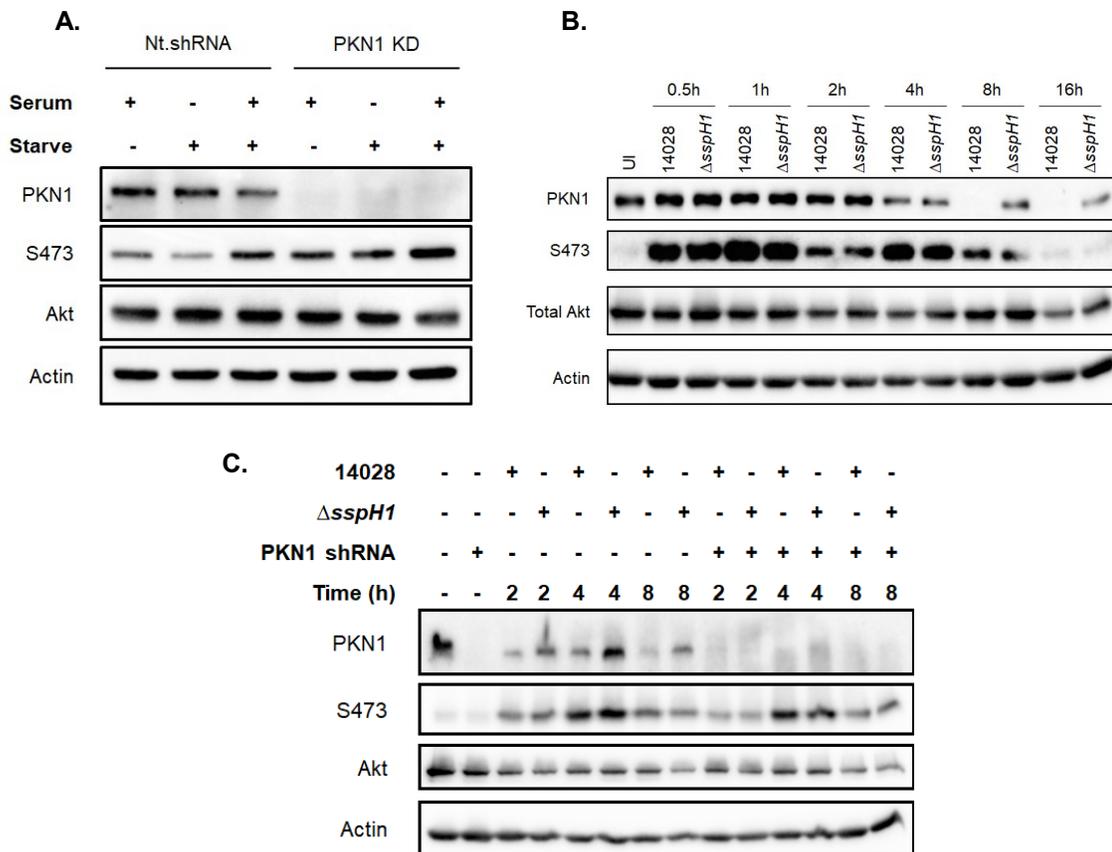
Figure 5.4: SspH1-dependent degradation of PKN1 does not impact Akt activation during *Salmonella* infection.
(A) PKN1 negatively regulates Akt activation under basal conditions. Immunoblot analysis using anti-PKN1, anti-Ser473 (S473) Phospho-Akt, and anti-Akt on U937 cells expressing PKN1 shRNA or non-targeting (Nt) shRNA with serum starvation (3 hours) and re-introduction of FBS. Actin immunoblots serves as loading control. Blot is representative of three independent experiments. (B) SspH1 does not impact Akt activation during *Salmonella* infection. Immunoblot analysis using anti-PKN1, anti-S473 Phospho-Akt, and anti-Akt on U937 cells infected with either wild-type (14028) or Δ*sspH1* at a MOI of 10. Lysates are from 0.5-, 1-, 2-, 4-, 8-, and 16-hours post-inoculation. Actin immunoblot serves as loading control. Blot is representative of three independent experiments. (C) Knockdown of PKN1 does not impact Akt activation during *Salmonella* infection. Immunoblot analysis using anti-PKN1, anti-S473 Phospho-Akt, and anti-Akt on U937 cells expressing PKN1 shRNA or Nt shRNA infected with either 14028 or Δ*sspH1* and a MOI of 10. Lysates are from 2, 4, and 8 hours post-inoculation. Actin immunoblots serves as loading control. Blot is representative of three independent experiments.

examined Akt activation in infection lysates from PKN1 KD and Nt shRNA U937 cells (Figure 5.4C). Again, a reduction of PKN1 in Nt shRNA U937 cells infected with wild-type did not affect p-Akt S473 levels relative to lysates from Δ*sspH1* infection. In PKN1 KD cells, p-Akt S473 levels were similar to those observed in Nt shRNA U937 cells, regardless of *Salmonella* strain use for infection. These results suggest that SspH1-dependent degradation of PKN1 does not impact Akt activation during *Salmonella* infection. Considering Akt activation contributes to intracellular replication of *Salmonella* (243), I conducted GPAs with wild type and Δ*sspH1* strains in U937, HeLa, and HT29 cells to verify the limited impact of PKN1 degradation on Akt phosphorylation during infection (Figure 5.5). The Δ*sspH1* mutant displayed similar levels of intracellular bacteria to wild type *Salmonella* at the 0-, 4-, and 18-hour time points in all three cell lines examined. Therefore, it is apparent that SspH1-dependent PKN1 degradation does not contribute to intracellular survival of *Salmonella*. Collectively, these results demonstrate that SspH1 does not regulate Akt activation through PKN1 degradation during *Salmonella* infection.

5.2.4. Distribution and Variance of *sspH1* in *Salmonella* Syst-OMICS Isolates

Reports on the distribution of *sspH1* among *Salmonella* serovars are inconsistent. While some groups report low *sspH1* prevalence in the *enterica* subspecies (412,539), others show high levels in *Salmonella* ser. Typhimurium (700–702). I was interested in determining the prevalence of *sspH1* within the *Salmonella* Syst-OMICS isolate consortium as a comparison to the current literature. In collaboration with Dr. Roger Levesque and colleagues, I screened whole genome sequences from the *Salmonella*
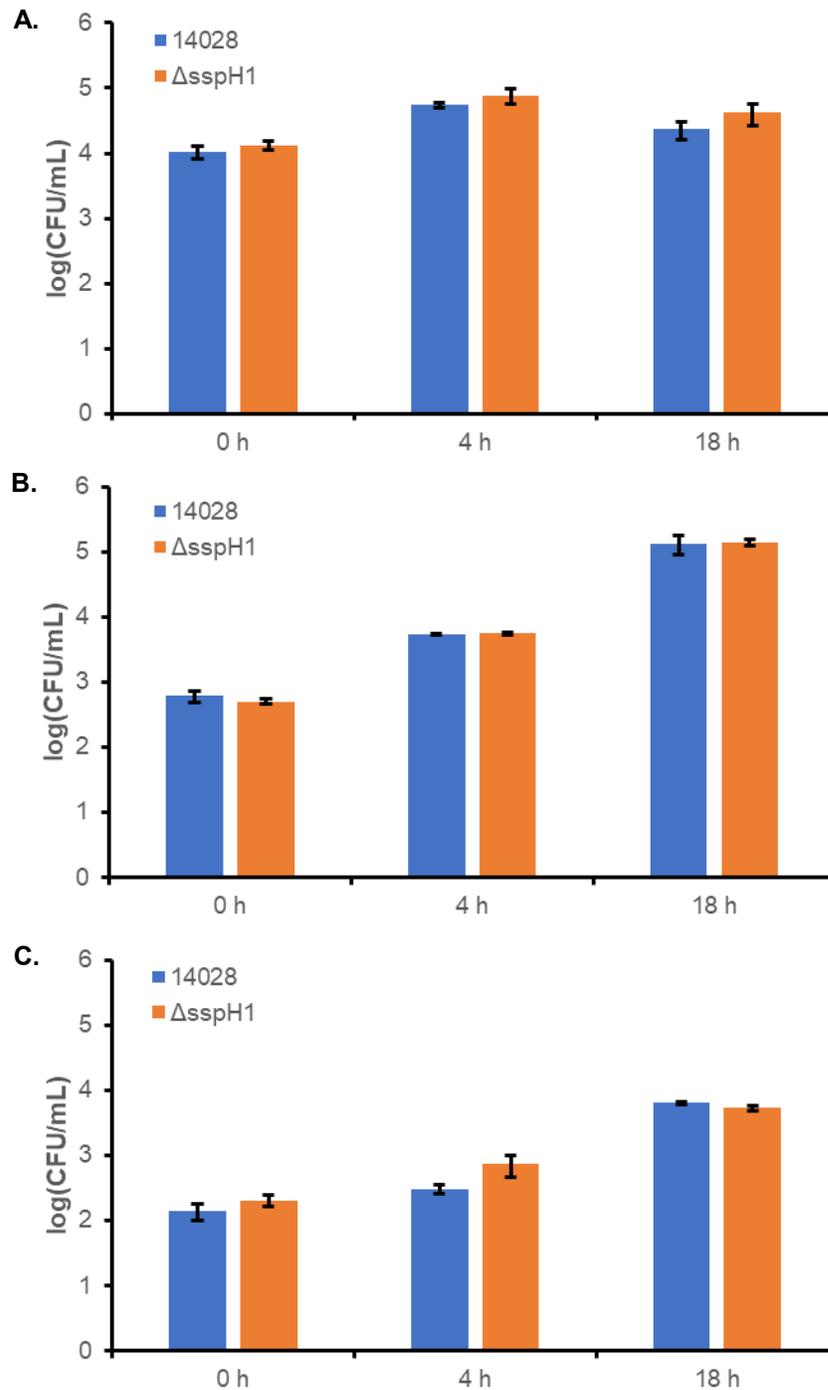
Figure 5.5: SspH1 is nonessential to *Salmonella* intracellular replication.
Enumeration of (log (CFU/mL)) of 14028 and Δ*sspH1* recorded at 0-, 4-, and 18-hours post-inoculation from gentamicin protection assays performed in U937 (A), HeLa (B), and HT29 (C) cell lines. Values are the means ± standard deviations of three independent experiments.

Foodborne Syst-OMICS (SalFos) database for the presence of *sspH1* in clinical and environmental *S. enterica* isolates (Figure 5.6). This screening identified 304 *Salmonella* isolates encoding *sspH1* from a total of 2,544 whole genome sequences (11.9%). Nearly all these isolates are classified as *S. enterica* subsp. *enterica*, with the lone exception identified under *S. enterica* subsp. *salamae*. From the *enterica* subspecies, Typhimurium is the predominant serovar encoding *sspH1* (21.7%), followed by Cerro (8.9%), and Javiana (8.6%) (Table 5.1). While Typhimurium isolates do not comprise a majority of the *sspH1* encoding serovars, these results suggest that *sspH1* is more prevalent in *S. enterica* subsp. *enterica* than initially thought.

In addition to variations in gene distribution, bacterial isolates commonly carry variable sequences or alleles for virulence factors (703,704). I was interested in evaluating whether *sspH1* varies greatly between *Salmonella* Syst-OMICs isolates. In collaboration with Dr. Roger Levesque and colleagues, I screened whole genome sequences from the SalFos database for SspH1-related proteins containing 50-85% amino acid similarity to evaluate *sspH1* sequence variation between *Salmonella* Syst-OMICS isolates (Figure 5.7). This screening identified 2,126 SspH1-related proteins from 2,544 *Salmonella* genomes. Clustering these proteins based on amino acid sequence similarity (>90%) generated 17 clusters of SspH1-related proteins. The largest cluster, cluster 1, corresponds to SspH2 with 1,477 similar proteins, followed by cluster 9 with 304 proteins representing SspH1. Clustering analysis identified a third major cluster, cluster 3, containing 236 similar proteins. Although, cluster 3 possesses approximately 65% amino acid sequence similarity to SspH1 and 75% sequence similarity to SspH2, it

Table 5.1: Number of *S. enterica* subsp. *enterica* isolates encoding *sspH1* from the *Salmonella* Syst-OMICS consortium classified by serovar.

S. enterica subsp. *enterica* serovar	# of isolates encoding *sspH1*	Percentage of total
Typhimurium	66	21.8%
Cerro	34	11.2%
Javiana	26	8.6%
Brandenburg	20	6.6%
Newport	15	5.0%
Alachua	13	4.3%
Saintpaul	12	4.0%
Paratyphi	10	3.3%
Ohio	10	3.3%
Senftenberg	8	2.6%
Miami	8	2.6%
Putten	7	2.3%
Bredeney	6	2.0%
Worthington	5	1.7%
Manhattan	5	1.7%
Sendai	4	1.3%
Sandiego	4	1.3%
Rubislaw	4	1.3%
Derby	4	1.3%
Livingstone	3	1.0%
Havana	3	1.0%
Hartford	3	1.0%
Florida	3	1.0%
Luciana	2	0.7%
Idikan	2	0.7%
Gaminara	2	0.7%
Duisburg	2	0.7%
Anatum	2	0.7%
TBD[1]	2	0.7%
Thompson	1	0.3%
Stanleyville	1	0.3%
Richmond	1	0.3%
Poona	1	0.3%
Panama	1	0.3%
Overschie	1	0.3%
Muenchen	1	0.3%
Mountpleasant	1	0.3%
Minnesota	1	0.3%
Hvittingfoss	1	0.3%
Hull	1	0.3%
Heidelberg	1	0.3%
Give	1	0.3%
Corvallis	1	0.3%
Bere	1	0.3%
Amsterdam	1	0.3%
Altona	1	0.3%
Agona	1	0.3%
Total	303	100%

[1]Antigenic classification to be determined (TBD)
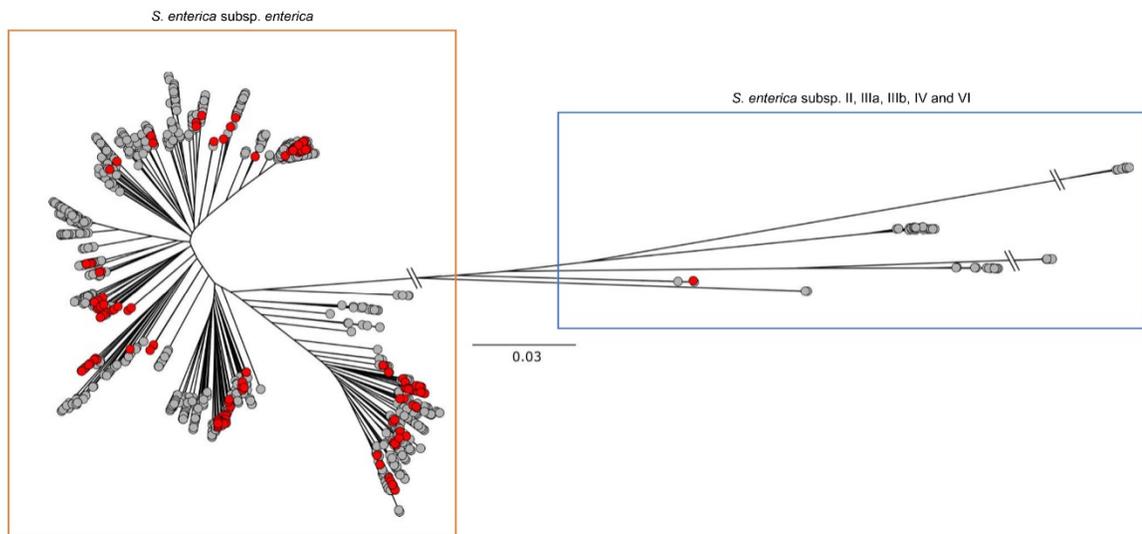
Figure 5.6: Distribution of SspH1 within *Salmonella* Syst-OMICS isolate consortium.

Unrooted maximum likelihood tree of 2,544 *S. enterica* genomes based on 173,657 SNPs using FastTree2.1.9. *S. enterica* genomes are separated based on subspecies, with *S. enterica* subsp. *enterica* in orange (2,500) and subsp. *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI) in blue (44). The 304 *S. enterica* genomes possessing the SspH1 virulence protein are identified in red.
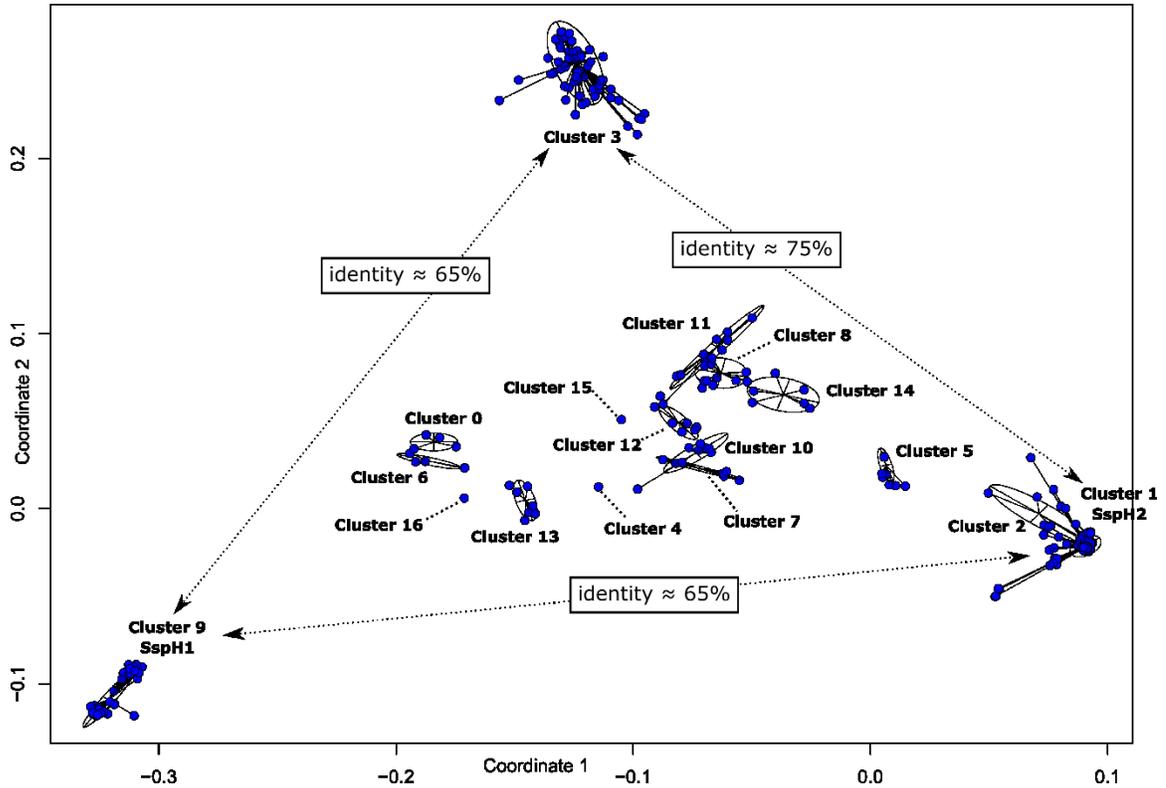
Figure 5.7: SspH1-related proteins encoded in *Salmonella* Syst-OMICS isolates
Multidimensional scaling of pairwise distances from aligned SspH1-related proteins using similarity. A total of 2,126 SspH1-related proteins were identified in 2,544 *Salmonella* genomes using BLASTp (identity ≥ 50% and coverage ≥ 85%). The proteins were clustered in 17 clusters using Uclust (identity = 90%) (cluster 0 (n=4), cluster 1 (n=1,477), cluster 2 (n=4), cluster 3 (n=236), cluster 4 (n=1), cluster 5 (n=19), cluster 6 (n=4), cluster 7 (n=12), cluster 8 (n=20), cluster 9 (n=304), cluster 10 (n=9), cluster 11 (n=10), cluster 12 (n=10), cluster 13 (n=8), cluster 14 (n=6), cluster 15 (n=1) and cluster 16 (n=1)).

represents a currently uncharacterized SspH1-related protein. Aside from the three major clusters, each of the remaining fourteen clusters contain twenty or less proteins with a collective total of 109. Altogether, these findings demonstrate some variation in SspH1-related proteins across *Salmonella* Syst-OMICs isolates and reveal a potentially uncharacterized *Salmonella* protein related to *Salmonella* NEL-type E3 ligases.

5.3. Discussion

Salmonella subverts various of host cell processes through the secretion of virulence effectors to promote infection. These effectors modulate a range of host enzymes and their activity to initiate internalization (705), support intracellular survival (261), and dampen host immune signaling (706). In this section, I examined the role of the E3 ubiquitin ligase effector SspH1 and its substrate PKN1 during *Salmonella* infection. Previous studies have shown SspH1 contributes to *Salmonella* disease progression in mammalian infection models, suggesting that the E3 ligase supports pathogenesis (412,673). My work demonstrates that SspH1 facilitates PKN1 degradation during *Salmonella* infection, confirming prior observations from transfection experiments (543). I hypothesized that SspH1 degrades PKN1 during *Salmonella* infection to promote pathogenesis. To answer this hypothesis, I investigated PKN1 and its contributions to regulating the pro-survival regulator Akt.

To study PKN1 in the context of *Salmonella* infection, it was important to identify a cell type that expresses high levels of PKN1. PKN1 expression varies across mammalian *in vitro* cell lines and is largely tissue-type specific (676,695,696). PKN1 expression is particularly high in lymphoid tissues where it is associated with leukocyte development and their migration (707,708). My work confirms these previous observations showing

that PKN1 is strongly expressed in the monocyte cell line U937 and is nominally expressed in the epithelial cells tested (Figure 5.2A). I elected to use U937 cells to study PKN1 during *Salmonella* infection due to its high PKN1 expression levels and because it models a cell type (macrophages) that commonly encounters *Salmonella* during GI invasion (42).

Research investigating potential substrates of bacterial E3 ubiquitin ligases typically uses chemical inhibition of the proteasome to demonstrate ubiquitin-associated degradation. For example, Li *et al.* recently used MG132 treatment on U937 cells during infection with *S. flexneri* to prove proteasomal degradation of GBP1 in response to IpaH9.8 ubiquitination (516). With respect to PKN1, inhibition of the proteasome is problematic as it upregulates activation of caspase-3 (699,709,710). Caspase-3 activation is typically observed during apoptosis and is responsible for cleaving PKN1 at three sites, producing three distinct apoptotic fragments (AFs) (690) (Figure 5.1). These PKN1 AFs retain their kinase activity, with AF3 exhibiting constitutively active kinase activity due to the removal of the autoinhibitory region (689). Caspase-3 cleavage of PKN1 also removes HR1b, which contains residues required for interaction with SspH1 (543). As a result, caspase-3 activation generates functional PKN1 fragments that are likely immune to SspH1 ubiquitination.

My work confirms that U937 treatment with MG132 reduces full length PKN1 levels (Figure 5.2B). It remains unclear whether this reduction is due to caspase-3 cleavage or by a different processing mechanism as the PKN1 antibody used for immunoblotting does not recognize PKN1 AFs. Alternatively, treatment with the pan-caspase inhibitor IDN-6556 increases levels of PKN1, presumably through the inhibition of caspase-3-

related cleavage. Several groups have used IDN-6556 to prevent caspase activation in response to pathogens (711,712). Seo and Rhee recently used IDN-6556 treatment in HeLa cells to prevent the cleavage of centrosomal proteins in response to MG132-related caspase-3 activation (713). My findings contrast those observed by Seo and Rhee as PKN1 levels are reduced with co-treatment of MG132 and IDN-6556 in U937 cells (Figure 5.2B). It is possible that PKN1 is subjected to caspase-3 independent processing or that higher dosing of IDN-6556 is required to off-set MG132-induced caspase-3 activation. However, *Salmonella* infection also stimulates caspase-3 activation during infection in macrophages (89), which when combined with MG132 treatment may hasten induction of apoptosis and amplify caspase-3-dependent cleavage. Rather than overstimulate caspase-3 activation during *Salmonella* infection with MG132, I opted to inhibit caspase cleavage with IDN-6556 to study PKN1 degradation.

Rohde *et al*. first demonstrated SspH1-dependent ubiquitination of PKN1 through *in vitro* ubiquitination experiments (512). Subsequent transfection experiments involving both SspH1 and PKN1 showed that PKN1 is degraded when co-expressed (543). My work demonstrates that SspH1 facilitates PKN1 degradation during *Salmonella* infection in macrophages (Figure 5.3A & C). However, ubiquitination is not solely responsible for this reduction in PKN1 levels as caspase-3 cleavage is also involved. *Salmonella* infection in macrophages stimulates caspase-3 activation through secretion of the T3SS-1 effector SipA and T3SS-2 effector SpvB (228,378). Furthermore, secretion of SspH1 is associated with suppressing NF-κB activity (235), which can increase caspase-3 activation (714). This infection stimulated caspase-3 activation likely contributes to PKN1 processing during *Salmonella* infection as PKN1 levels decreased over time in

both 14028 and Δ*sspH1* infected cells (Figure 5.3A & C). To inhibit caspase activation, I blocked the effects of caspase-3 cleavage to PKN1 processing during infection through pre-treatment of U937 cells with IDN-6556. Consequently, PKN1 levels were higher in caspase inhibited cells relative to DMSO controls (Figure 5.3B). More importantly, wild type infected U937 cells exhibited lower levels of PKN1 relative to Δ*sspH1* indicating that SspH1 ubiquitination is also responsible for the reduction in substrate levels. The increase in molecular weight observed for PKN1 in each infection condition is likely attributed to host-initiated ubiquitination of PKN1 in response to activation (715,716). Additional experiments involving infections in a Δ*sipA*Δ*spvB* background to lower caspase-3 activation and assessment of caspase-3 activity in infection lysates would assist in further separating the contributions of caspase-3 cleavage to PKN1 processing.

The role of PKN1 during *Salmonella* infection is largely understood in the context of SspH1-associated phenotypes. Outside of infection, PKN1 activity is associated with negatively regulating NF-κB activity (542,717). SspH1 is also implicated in downregulating NF-kB activity and expression of pro-inflammatory cytokines (542). However, transfection experiments have demonstrated that SspH1 downregulates NF-κB activity independent from its E3 ligase activity and PKN1 binding, suggesting an alternative mechanism of regulation (543). PKN1 is also involved in potentiating steroid hormone receptor activity, specifically for androgen receptor (AR), mineralocorticoid receptor (MR), and progesterone receptor (PR) signaling (682). Co-transfection experiments of SspH1 and PKN1 demonstrated that SspH1 E3 ligase activity suppresses AR signaling through degradation of PKN1 (543). How SspH1-related AR suppression

impacts *Salmonella* infection is unclear; although, AR signaling is important to neutrophil development and function (718).

Aside from regulating NF-κB and AR activity, PKN1 is also involved in suppressing Akt and the pro-survival pathway. In B cells, PKN1 interacts with Akt and suppresses its kinase activity (693). This suppression is linked to the kinase domain of PKN1 as expression of PKN1 AF3 is sufficient to impair PDK1-dependent phosphorylation of Akt (692). PKN1 AF3 is also generated in response to apoptosis suggesting PKN1 kinase activity promotes pro-apoptotic signaling (690). Furthermore, *Pkn1* knockouts in mice and cerebellar granule cells display an increase in basal p-Akt S437 (693,694). However, knock-in of kinase dead *Pkn1* is not associated with an increase in basal p-Akt S473 in mice (708). My work demonstrates that knockdown of PKN1 in U937 cells increases p-Akt S473 levels at basal conditions, during serum starvation, and following serum re-introduction (Figure 5.4A). Acute serum starvation and subsequent serum re-introduction are conditions used to suppress and stimulate Akt phosphorylation, respectively (719). Although p-Akt levels are higher in PKN1 KD cells for all three conditions relative to the Nt shRNA control, it remains unclear whether PKN1 is specifically responsible for regulating basal Akt activation or activation in response to various stimuli.

Regarding *Salmonella* infection, Akt plays a pivotal role in determining host cell fate and bacterial replication. *Salmonella* regulates Akt activation via secretion of the T3SS-1 effector SopB, which remodels the PIP landscape at the plasma membrane (244,720). Without SopB secretion, *Salmonella* fail to activate Akt and infected cells are subjected to early apoptosis (242). In addition to promoting cell survival, Akt activity

also supports intracellular replication as macrophages treated with chemical Akt inhibitors exhibit lower intracellular levels of *Salmonella* (721). Additionally, Akt plays a protective role during *Salmonella* mouse infections as Akt knockout mice display higher bacterial burdens and increased inflammatory pathology at the site of invasion (722). This increase in intestinal damage is linked to compromised epithelial barrier integrity as Akt promotes tight junction expression in epithelial cells (723).

Considering the importance of Akt signaling to *Salmonella* infections, I examined whether SspH1-dependent PKN1 degradation affects Akt activation in macrophage infection (Figure 5.4B & C). My work demonstrates that neither SspH1-dependent PKN1 degradation nor PKN1 knockdown influences Akt phosphorylation during *Salmonella* infection. Moreover, SspH1 does not contribute to intracellular replication in macrophages and epithelial cells supporting its detachment from Akt regulation (Figure 5.5). It is unsurprising that SspH1-dependent PKN1 degradation alone fails to impact Akt activation as SopB activity stimulates approximately 90 host kinases that regulate Akt phosphorylation (724). At the very least, these findings highlight the complex network that regulates the Akt pro-survival pathway during *Salmonella* infection.

Despite numerous scientific investigations, the impact of SspH1 on *Salmonella* infection remains ambiguous. Our limited understanding of how *sspH1* contributes to infection is likely a reflection of its minimal distribution among disease-causing serovars. Early studies involving *sspH1* discovered that the E3 ubiquitin ligase is sparingly distributed among *S. enterica* subsp. *enterica* serovars and is instead primarily found in *S. enterica* subspecies associated with amphibians and reptiles (412,539). However, recent studies characterizing clinical and environmental *Salmonella* isolates show that *sspH1* is

found in most *Salmonella* ser. Typhimurium strains (700–702). Work I completed in collaboration with the Dr. Roger Levesque and colleagues confirms these recent studies that *sspH1* is mainly found in isolates classified under the *enterica* subspecies (Figure 5.6); although, the analysis is limited by the inclusion of relatively fewer non-*enterica* subspecies isolates (44 isolates to 2500). Classifying these isolates by serovar reveals that serovar Typhimurium possesses the highest *sspH1* prevalence (Table 5.1). This serovar analysis confirms recent studies reporting high prevalence of *sspH1* in *Salmonella* ser. Typhimurium isolates (700–702). Given that *Salmonella* ser. Typhimurium is a leading contributor to *Salmonella* illness in North America (57,546), a major question emerges, what fitness benefit does SspH1 provide? Additional research studying SspH1 during infection is required to answer this question.

My work involving the characterization of SspH1 during infection focuses entirely on the *sspH1* gene from the *Salmonella* ser. Typhimurium laboratory strain 14028. Research involving this version of *sspH1* may not reflect *sspH1* variants that are present across *Salmonella* isolates as some *Salmonella* virulence factors display a wide range of alleles. For example, nucleotide sequences for the E3 ubiquitin ligase SspH2 are highly variable between *S. enterica* subsp. *enterica* serovars (725). Unlike SspH2, the sequence variation of SspH1 between *Salmonella* isolates is currently unknown. Work I completed in collaboration with Dr. Roger Levesque and colleagues identified 17 unique clusters of SspH1-related proteins (Figure 5.7). As expected, clustering analysis identified SspH2 as the largest SspH1-related protein group as *sspH2* is found in a majority of *Salmonella* isolates and contains 69% nucleotide sequence similarity to *sspH1* (412). Surprisingly, clustering analysis identified a major SspH1-related protein cluster (cluster 3) that

represents a currently uncharacterized *Salmonella* protein. This protein cluster possesses higher amino acid sequence similarity to SspH2 (~75%) relative to SspH1 (~65%). Previous studies have detected SspH2-like proteins in *S. enterica* subsp. *arizonae* (412) and *sspH2* pseudogenes in subspecies *enterica* serovars (725,726). It remains to be determined if the SspH1-related protein of cluster 3 matches either of these previously discovered SspH2 variants. Clustering analysis also identified 14 additional SspH1-related proteins with varying degree of protein sequence similarity to SspH1. Further analysis into the NEL and LRR domains of these SspH1-related proteins may provide insight into the functionality of their substate specificity and E3 ligase activity.

The work presented in this section on SspH1 confirms that the E3 ubiquitin ligase facilitates degradation of the host kinase PKN1 during *Salmonella* infection. I report that both SspH1 and caspase-3 activation contribute to PKN1 processing during infection in macrophages. Outside of *Salmonella* infection, PKN1 is involved in regulating basal Akt activation. However, SspH1-dependent PKN1 degradation during *Salmonella* infection fails to impact Akt phosphorylation. My work also confirms the high prevalence of *sspH1* in *Salmonella* ser. Typhimurium isolates and potentially identifies a new SspH1-related protein. Collectively, these findings highlight the complexity of SspH1 the cellular processes it regulates during *Salmonella* infection.

Chapter 6: Conclusions, Limitations, and Future Directions

6.1. Conclusions and Limitations

Although the work presented in this thesis provides insight into molecular, cellular, and food safety aspects of *Salmonella*, it also raises many questions. In chapter 3, I hypothesized that *Salmonella* is a major contributor to microbial-related food recalls in Canada. My work demonstrated that *Salmonella* is responsible for the largest number of microbial-related food recall event in Canada from 2000-2017. Additionally, *Salmonella*-related recalls are increasing for the specific food products, such as vegetables, leafy greens, and fruit. However, this work is limited to representing suspected contamination as not all recalls are initiated in response to the confirmed presence of pathogens. This work is further limited by the biases I introduced in classifying food items by United States FDA industry codes as the CFIA does not classify recalls by product types. Despite these limitations, this work still shows that *Salmonella* substantially contributes to recalls in Canada. As rates of *Salmonella*-related recalls increase in Canada, what improvements to food safety can be made to mitigate the risks of future recall-related outbreaks?

In chapter 4, I hypothesized that a protozoan infection model could distinguish between virulent and avirulent *Salmonella* isolates. My work demonstrated the utility of *Acanthamoeba* spp. as an infection model for screening *Salmonella* isolates for virulence. However, there are two main limitations of this model. First, the model only evaluates the degree of internalization into amoeba, while *Salmonella* pathogenesis involves multiple processes following entry. Second, the two references strains selected as standards for the screening have not been previously evaluated in an amoeba infection model. Inclusion of

more controls, such as *Salmonella* strains or other bacteria with documented predation susceptibility, could serve as additional references for screened isolates. Considering these limitations, what other assays and controls could be used to evaluate the pathogenic potential of *Salmonella* isolates using the *Acanthamoeba* infection model?

Lastly, in chapter 5, I hypothesized that SspH1 degrades PKN1 during infection to enhance *Salmonella* pathogenesis. My work demonstrated that SspH1 facilitates PKN1 degradation during *Salmonella* infection in macrophages and that caspase activation appears to impact PKN1 processing. However, there are two main limitations to these findings. First, I could not distinguish between PKN1 cleavage and PKN1 degradation in immunoblots as the PKN1 antibody I used recognizes an epitope that is absent in PKN1 cleavage products. Second, aside from pan-caspase inhibition, I did not examine caspase activation during *Salmonella* infection. Consequently, a different PKN1 antibody and additional experiments assessing caspase activation during *Salmonella* infection may be required to distinguish the contributions of SspH1 and caspase activation to PKN1 processing. My work also demonstrated that SspH1-dependent PKN1 degradation does not impact Akt activation during *Salmonella* infection. How PKN1 degradation contributes to *Salmonella* pathogenesis is still unclear. It is possible that SspH1 cooperates with additional *Salmonella* effectors to subvert host signaling for intracellular survival. Furthermore, SspH1 and other SspH1-related proteins may interact with additional host substrates other than PKN1. Exploring these unresolved questions form the basis of the final chapter of my thesis.

6.2. Improvements to Microbial-Related Recalls Involving *Salmonella* Contamination

In chapter 3, I evaluated the contributions of *Salmonella*-related food recalls reported by the CFIA from 2000-2017 relative to other major foodborne pathogens. While this work highlights the substantial number of *Salmonella*-related food recalls in Canada, it does not truly represent the amount of food recalls caused by *Salmonella* contamination. Not all microbial-related food recalls are initiated in response to detection of a foodborne pathogen as some recalls are triggered based on speculated contamination. Consequently, the findings from this research serve more as an estimation of *Salmonella* contamination. However, these estimates do not diminish the risk associated with *Salmonella*-related recalls.

In 2018, endemic transmission (i.e. mishandling or consumption of raw meat) accounted for 54% of reported *Salmonella* illness in Canada, whereas foodborne outbreaks linked to recalled food items were responsible for 12% (57). To evaluate the prevalence of *Salmonella* contamination in food, the Public Health Agency of Canada (PHAC) performs routine surveillance on meat products, animals, manure, and agriculture irrigation. There are currently minimal surveillance measures in Canada regarding food items commonly linked to *Salmonella* recalls, such as nuts/edible seeds, spices, and produce. The United States FDA, however, regularly surveys contamination in these foods (727–729). Although *Salmonella* prevalence rates are lower for nuts/edible seeds, spices, and produce relative to meats, their risk to food safety is growing as *Salmonella*-related recalls increase (Figure 3.2C). Therefore, it may be in the best interest of the CFIA and PHAC to increase surveillance on food items most associated with

Salmonella-related recalls to obtain a better measure of *Salmonella* contamination in Canada.

Salmonella-related recall surveillance in Canada can also improve with the implementation of WGS technologies. As of 2017, PHAC adopted WGS as the primary method for subtyping *Salmonella* isolates, abandoning less discriminatory techniques such as PFGE and MLTV (730). PulseNet Canada, the national molecular subtyping network for foodborne disease surveillance, now solely uses WGS subtyping techniques such as single nucleotide variant (SNV) and whole genome MLST (wgMLST) analysis to cluster bacterial strains isolated during foodborne outbreaks (731,732). In addition to enhancing the discriminatory power of subtyping, WGS provides insight into the antimicrobial resistance and virulence factor profiles of isolates. For example, recent research from Health Canada demonstrates ~95% accuracy in antimicrobial resistance predictive modeling of *Salmonella* isolates using WGS analysis (733). In contrast to PHAC, the CFIA currently performs minimal subtyping of *Salmonella* strains isolated from contaminated food products. Adopting WGS analysis of *Salmonella* isolates from contaminated food products would assist in identifying strain types that commonly contaminate specific foods. More importantly, WGS analysis of *Salmonella* isolates would support linking strains isolated from contaminated food to cases in foodborne outbreaks.

One final improvement that should be made to microbial-related recalls in Canada involves the classification of product types. The United States FDA categorizes food recalls by industry codes to group recalls involving similar product types together (555). Conversely, the CFIA does not use industry codes to classify reported food recalls. As a

result, I classified microbial-related food recalls in Canada from the examined period using the industry code categories defined by the United States FDA. In doing so, I introduced my own biases in interpreting food product types for each microbial-related food recall. In the future, I recommend that the CFIA adopts classification of food recalls by FDA product codes, or implementation of a similar product codex, to improve recall trend analysis.

6.3. Exploring the *Acanthamoeba* Screening Model to Characterize *Salmonella* Virulence

In chapter 4, I demonstrated the effectiveness of *Acanthamoeba* spp. as an infection model for characterizing internalization phenotypes for *Salmonella* isolates. The purpose of this work was to develop an efficient screening method to evaluate the virulence potential of *Salmonella* isolates using protozoa. While internalization is important to *Salmonella* as an intracellular pathogen, it is only one of several processes that contribute to pathogenesis. The *Acanthamoeba* model can assess additional aspects of bacterial pathogenesis as evident by its extensive application in studying *L. pneumophilia* (153,734–736)*, Mycobacterium* spp. (737–740), and *L. monocytogenes* (741–744). Many of the methods used in these studies are also applicable to examining *Salmonella* virulence in further detail.

Following internalization, the next logical step of pathogenesis to examine in the *Salmonella* Syst-OMICS isolate consortium is intracellular survival. Several studies have investigated the contributions of specific *Salmonella* virulence factors to intracellular survival in *Acanthamoeba*. For instance, deletions mutants of T3SS-2 and the two-component regulatory system PhoPQ both significantly impair intracellular survival in amoeba (181). However, outside of these virulence factors, the genes responsible for

intracellular survival within *Acanthamoeba* are largely unidentified. To effectively evaluate intracellular survival of the *Salmonella* Syst-OMICS isolates, I would include mutant strains with known defects in survival, such as *phoP* and *sseB*, to serve as reference phenotypes. I would also include the laboratory *E. coli* strain DH5α as a negative control as it is commonly used to feed amoeba in non-nutrient buffer (745). Given the number of isolates and tedious nature of GPAs, it is in the best interest of efficiency to examine only a single intracellular survival time point in addition to the initial time point required to quantify internalized bacteria. If the internalization screening is any indication, I would expect a range of intracellular replication phenotypes among the *Salmonella* Syst-OMICS isolates.

A second screening that would provide insight into *Salmonella* virulence is the evaluation of amoeba viability. Similar to macrophages, *Salmonella* infection induces apoptosis-like cell death in *Acanthamoeba* (182). While my amoeba viability experiments showed no difference between Δ*invA*Δ*sseB* and the parental strain (Figure 4.4B), I used a lower MOI (10) relative to previous research (100) (182). It is also possible that neither T3SS-1 nor T3SS-2 contribute to lethality in amoeba as there is minimal evidence regarding *Salmonella*-induced cell death in *Acanthamoeba*. To improve our understanding of *Salmonella*-induced amoeba cell death, I propose to screen amoeba viability in response to each *Salmonella* Syst-OMICS isolate. The evaluation of amoeba viability is amenable to high-throughput screening either through alamarBlue microplate experiments (746) or Annexin-V/PI double staining flowcytometry (747). These experiments would include DH5α as a negative control and a *phoP* mutant as a reference strain, which exhibits minimal induction of apoptosis during *Salmonella* infections in

macrophages (748). Given that previous investigation into *Salmonella*-induced amoeba cell death showed differences between *S. enterica* serovars (182), I would expect a range of lethality phenotypes among the *Salmonella* Syst-OMICS isolates.

The final potential screening model involving the *Salmonella* Syst-OMICs isolate consortium involves *Acanthamoeba* grazing assays. Although the grazing experiments I performed in *A. castellanii* displayed no difference between Δ*invA*Δ*sseB* and the parental strain (Figure 4.3), these assays represent only one method of measuring predation resistance. Grazing assays conducted in non-nutrient buffer can be extended across multiple days to examine *Acanthamoeba* cyst formation in the presence of bacteria (749). Previous research demonstrates that *Salmonella* survival within amoeba cysts for up to three weeks and are protected from acidic conditions and bactericidal concentrations of antibiotics (666). Considering the high prevalence of amoeba in the environment (173,750,751), cysts represent a potential shelter and survival niche for foodborne pathogens. Identifying *Salmonella* genetic factors that contribute to intracystic survival may help inform future mitigation food safety strategies. To evaluate intracystic survival of the *Salmonella* Syst-OMICS isolates, I would conduct extended grazing assays in non-nutrient buffer with similar negative controls to previously mentioned screens (DH5α and Δ*phoP*) and quantify intracellular bacteria following cyst lysis. I would expect a similar range of phenotypes to the intracellular survival screen since they evaluate related cellular processes.

6.4. Exploring the Role of PKN1 in *Salmonella* pathogenesis

In chapter 5, I demonstrated that SspH1 facilitates PKN1 degradation during *Salmonella* infection in macrophages (Figure 5.3). Outside of *Salmonella* infection,

PKN1 regulates Akt activation as demonstrated by my work (Figure 5.4A) and others (692–694). However, PKN1 degradation during *Salmonella* infection in macrophages does not impact Akt phosphorylation (Figure 5.4B & C). As PKN1 is involved in regulating several cellular processes (674), additional research is required to identify how SspH1-dependent PKN1 degradation influences *Salmonella* pathogenesis.

PKN1 is a proven regulator of AR, PR, and MR signaling activity and directly interacts with AR (682). Work by Sicheri and colleagues revealed that SspH1-dependent degradation of PKN1 impairs AR signaling via transfection experiments (543). However, this suppression of AR signaling has not been confirmed during *Salmonella* infection. I propose to evaluate AR signaling in U937 and HT29 cells during infection with either wild type or Δ*sspH1* in the presence or absence of the synthetic androgen R1881 (Figure 6.1A). I would specifically measure transcription of AR-regulated genes (752) and monitor localization of fluorescently tagged AR (753) in response to infection. If SspH1-dependent PKN1 degradation is responsible for suppressing AR activity, I would expect increased transcription of AR regulated genes and localization of AR at the nucleus in infection with Δ*sspH1* relative to wild type. Following these experiments, I would perform infections in AR knockout mice (718) with wild type and Δ*sspH1* and examine bacterial burdens, intestinal pathology, and immune cell infiltrate to gain further insight into the role of SspH1 on *Salmonella* pathogenesis.

PKN1 also interacts with and phosphorylates mixed lineage kinase (MLK)-like mitogen-activated protein triple kinase (MLTK), a MAPKKK family member, of the p38 signaling pathway (684). Research regarding PKN1 regulation of MLTK and p38 is
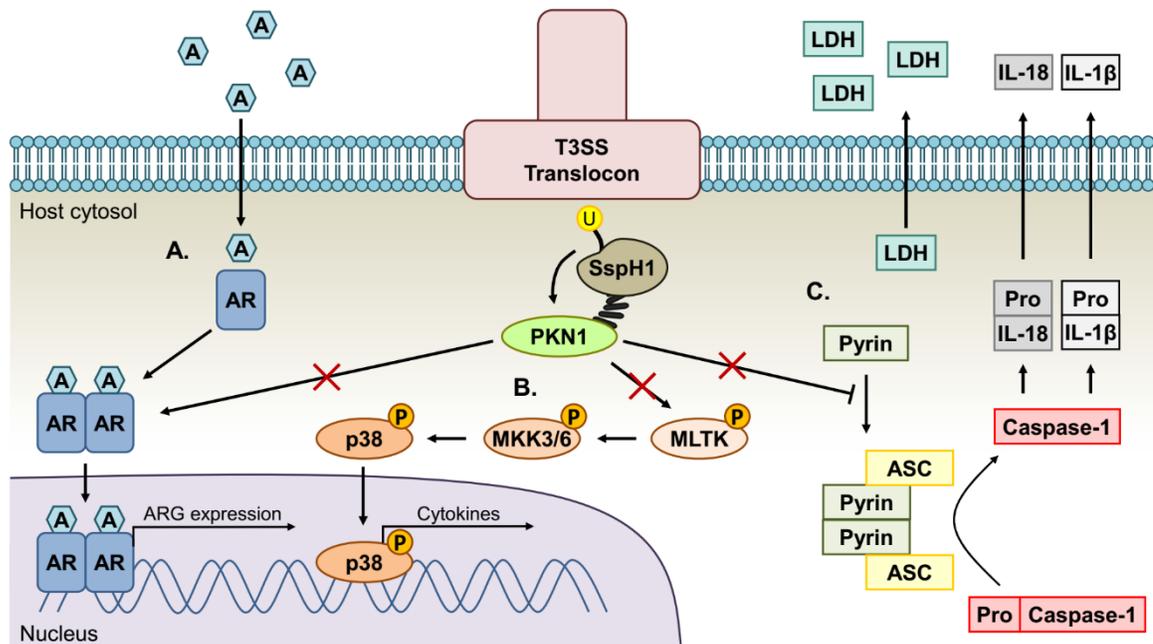
Figure 6.1: Potential roles of SspH1 during *Salmonella* infection.
The role of SspH1 during *Salmonella* infection is likely linked to its impact on PKN1 degradation. PKN1 governs several cellular processes, some of which may contribute to *Salmonella* infection. (A) PKN1 binds androgen receptors (ARs) and potentiates their signaling in response to binding androgens, such as testosterone. ARs dimerize upon activation and migrate to the nucleus where they upregulate the expression of androgen-regulated genes (ARGs). In the absence of PKN1, AR signaling substantially diminishes. Therefore, SspH1-dependent PKN1 degradation may suppress ARG expression during infection. (B) PKN1 binds the host kinase MLTK and promotes activation of the p38 signaling pathway. *Salmonella* infection activates p38 signaling leading to increased expression of cytokines. SspH1 is associated with downregulating expression of pro-inflammatory cytokines. Therefore, SspH1-dependent PKN1 degradation may suppress MLTK and p38 activation during *Salmonella* infection. (C) PKN1 binds and phosphorylates the host protein pyrin, preventing its dimerization and interaction with apoptosis-associated speck-like protein containing a caspase recruitment domains (ASCs). Without phosphorylation, the ASC-pyrin complex facilitates pro-caspase-1 cleavage, which stimulates inflammasome activation and pyroptosis. Pyroptosis is characterized by expression of the pro-inflammatory cytokines IL-1β and IL-18, and leakage of lactate dehydrogenase (LDH) from the compromised plasma membrane. *Salmonella* thrive under inflammatory conditions within the GI tract. Therefore, SspH1-dependent PKN1 degradation may facilitate inflammasome activation to benefit *Salmonella* during infection.

inconsistent as some studies show that PKN1 KD does not impact p38 activation (693,754), whereas others show PKN1 activity promotes p38 signaling (677,683,684,755). During infection, *Salmonella* effector-associated Rho-GTPase activation stimulates p38 signaling, subsequently upregulating expression of pro-inflammatory cytokines (217). Moreover, chemical inhibition of p38 signaling during infection is associated with an increase in intracellular replication in macrophages (756) and a reduction in IL-8 secretion in epithelial cells (757). It is currently unknown whether PKN1 degradation during *Salmonella* infection influences p38 signaling. I propose to evaluate p38 activation in PKN1 KD and mock control U937 and HT29 cells infected with either wild type *Salmonella* or the Δ*sspH1* strain (Figure 6.1B). If PKN1 is involved in regulating p38 activation during infection, I would expect increased p38 phosphorylation in Δ*sspH1* infected cells. I would then measure transcription of p38-regulated pro-inflammatory cytokines (758) and monitor nuclear localization of fluorescently tagged p38 (759) in response to infection. The results from these experiments could improve our understanding of previous findings regarding SspH1-dependent suppression of infection-induced pro-inflammatory signaling (235).

In addition to regulating the Akt pro-survival pathway, PKN1 activity governs pyrin-associated inflammasome activation. PKN1 specifically phosphorylates pyrin, which prevents inflammasome formation and subsequent cleavage of pro-caspase-1 (685). The LRR-containing effector YopM of *Yersinia* spp. modulates PKN1 to phosphorylate pyrin and prevent inflammasome activation stimulated by infection in macrophages (527). In *Salmonella*, T3SS-1 and LPS are associated with inducing inflammasome activation and pyroptosis in macrophages (227,760). Given that SspH1 is

a T3SS-1 effector and PKN1 activity suppresses inflammasome activation, it is reasonable to consider that SspH1 is involved in regulating pyroptosis (Figure 6.1C). To investigate this potential regulation of inflammasome activation, I propose to compare inflammasome markers (IL-1β, IL-18, and caspase-1) between U937 cells infected with either wild-type *Salmonella* or the Δ*sspH1* strain in PKN1 KD and mock control backgrounds. I would also monitor lactate dehydrogenase (LDH) release from these infections as it is a common indicator of pyroptosis (761). If PKN1 does regulate inflammasome activation during infection, I would expect lower levels of inflammasome markers and LDH release in infections with Δ*sspH1*. While it may seem counterintuitive for an effector like SspH1 to promote inflammasome activation, pyroptosis promotes inflammation (762), which increases accessibility of carbon sources and expression of toxins that support *Salmonella* replication over commensal bacteria (262,763,764). A similar mechanism is demonstrated by *S. flexneri* via the secretion of the NEL-type E3 ligase effector IpaH7.8, which promotes inflammasome activation in macrophages through ubiquitination and degradation of GLMN (520).

6.5. Investigate *Salmonella* Effector Cooperation with SspH1

A substantial degree of regulatory overlap exists among the diverse repertoire of *Salmonella* effectors. For example, T3SS-1 effectors SopB, SopE, and SopE2 all modulate activity of Rho-GTPases to facilitate *Salmonella* internalization (212,705). Furthermore, T3SS-2 effectors SopD2 and GtgE work collectively to impair Rab-associated membrane trafficking to the SCV (404). By comparison, very little is known regarding the regulatory overlap of *Salmonella* effectors with SspH1 and PKN1 degradation. Outside of infection, PKN1 is involved in membrane trafficking and

localizes to endosomes in response to cell stress (680). PKN1 also interacts strongly with the Rho-GTPases RhoA and RhoB and is activated in response to phosphatidylinositol (3, 4, 5) triphosphate (PI(3, 4, 5,)P$_3$) generation (679,686,688,765). During infection, the T3SS-1 effector SopB facilitates the generation of PI(3, 4, 5)P$_3$ at the SCV and the recruitment of RhoB to the plasma membrane (211,244), while the T3SS-2 effector SseJ recruits and binds RhoA at the phagosome surface (283). Whether SspH1-dependent regulation of PKN1 is influenced by this activity during *Salmonella* infection remains to be determined. SspH2 may also cooperate with the regulatory activities of SspH1 as both are required to produce fatal illness in a calf infection model (412).

To evaluate *Salmonella* effector cooperation with SspH1, I would screen transposon (Tn) mutant libraries from wild type and Δ*sspH1* backgrounds through the streptomycin treated mouse model (Figure 6.2). In this model, pools of mutants from each individual background are inoculated into mice and are recovered from the spleen following five days of infection (766). Tn sequencing (Tn-seq) analysis is then performed on recovered mutants using deep sequencing to identify and quantify insertion sites. This analysis provides a measurable output of virulence as genes containing minimal or no insertions are considered important to the selective pressure (767). With respect to the two separate *Salmonella* backgrounds, genes lacking insertions in the Δ*sspH1* screen, but not in the wild-type background, may encode proteins that cooperate with SspH1 during infection. I would then create double mutants with *sspH1* and the candidate genes and characterize their defects in virulence in the mouse model. Previous studies have employed Tn-seq to define new gene functions in *Salmonella* with success (768–771). At the very least, I would confirm or disprove the cooperation between SspH1 and SspH2.
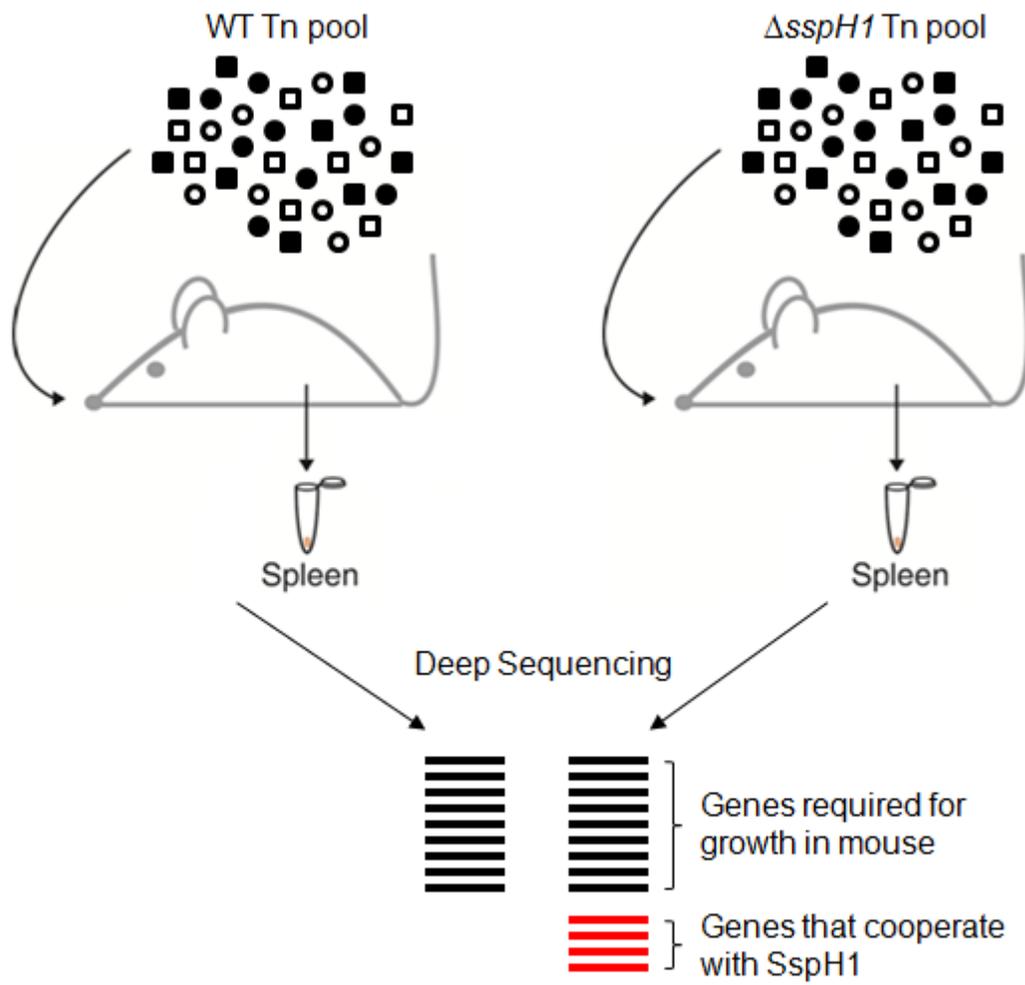
Figure 6.2: Identification of *Salmonella* genes that cooperate with SspH1
A large pool of transposon (Tn) mutants is generated in wild type *Salmonella* and *Salmonella* deleted for *sspH1*. Pools of mutants are used to orally infect mice. After five days, bacteria are recovered from spleens. Deep sequencing analysis is performed on recovered bacteria pools. Mutants that are not detected in recovered bacteria are characterized as lacking a gene required for growth in mice. Genes classified as required for growth in *sspH1* background infections, but not wild type background infections, are analyzed as candidates for cooperation with SspH1. *Salmonella* proteins that cooperate with SspH1, such as SspH2 and potentially others, would be identified using this approach.

6.6. Identification of SspH1 and SspH1-like Protein Substrates

Miller and colleagues first identified the interaction between SspH1 and PKN1 via a yeast-two hybrid screen (542). Several groups later confirmed this interaction through *in vitro* ubiquitination assays, x-ray crystallography, and affinity-purification mass spectroscopy (AP-MS) (512,543,544). While PKN1 is the only host substrate currently identified for SspH1, alternative targets for SspH1 may also exist as E3 ligases are unlikely to be restricted to a single substrate. For example, the *Salmonella* NEL-type E3 ligase SlrP possesses two known substrates in Trx and ERdj3 (534,535). Moreover, recent work demonstrates that IpaH9.8 of *S. flexneri* ubiquitinates multiple GBP types in addition to its interaction with NEMO (513,518). To identify new potential substrates for SspH1, I propose three different approaches with well documented use in screening for E3 ubiquitin ligase interactions.

The first approach involves the ubiquitin activated interaction trap (UBAIT) system (Figure 6.3). Briefly, an E3 ligase is cloned into a vector so that a tripartite fusion protein is expressed: an N-terminal epitope tag that can be purified from cell lysates, the E3 ligase, and a C-terminal ubiquitin. The ubiquitin is recognized and activated by E1 enzymes and charged by E2 enzymes. This E3-ubiquitin fusion protein then interacts with substrates to form a covalently modified complex. These complexes are subsequently purified using the epitope tag and analyzed by mass spectrometry to identify potential substrates (772). I would modify this system to express an 3X FLAG-SspH1-ubiquitin fusion protein in *Salmonella* to generate E3-ubiquitin-substrate complexes during infection (Figure 6.3). The use of UBAIT during infection, rather than transient
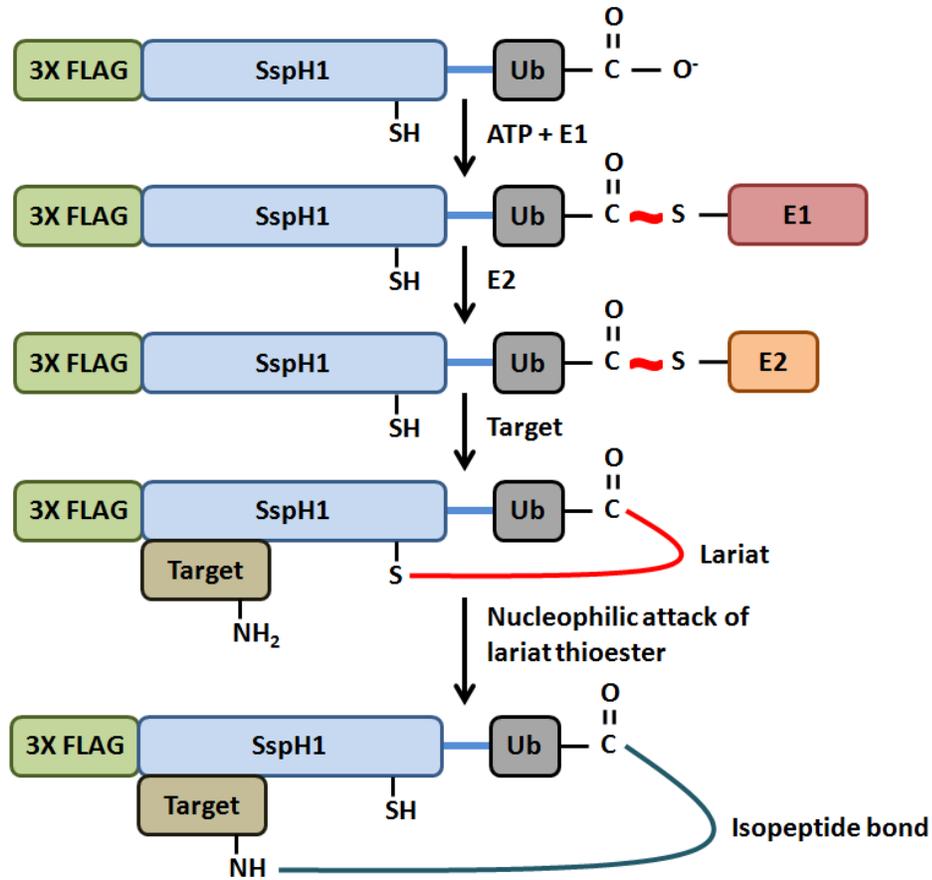
Figure 6.3: UBAIT strategy to identify SspH1 targets.
SspH1 is fused to ubiquitin and a 3X FLAG epitope. An E1 enzyme facilitates the formation of a thioester-linked complex. The complex is then transferred to an E2 enzyme where the catalytic cysteine of the E3 enzyme SspH1 attacks the E2 thioester, forming a thioester-linked protein lariat structure. In host cells, SspH1 substrates bind to the LRR domain of the E3 ligase. Substrate lysine residues in close proximity attack the thioester bond, forming an isopeptide linkage between the substrate and the UBAIT construct. The resulting complex is then purified via the 3X FLAG epitope. Figure adapted from O'Connor *et al.*, 2015 (773).

transfection, would minimize false positives that can arise due to overexpression and would potentially identify metaeffector activity (774), where SspH1 might ubiquitinate other *Salmonella* secreted effectors. I would also construct a UBAIT-SspH1 fusion protein with non-conjugatable ubiquitin to serve as a negative control for ubiquitinated substrates. Based on previous success with the UBAIT system (773), I would expect at the very least that this strategy would confirm the interaction between SspH1 and PKN1.

The second approach involves the proximity-dependent biotin identification (BioID) system. This approach is based on the biotin ligase protein BirA derived from *E. coli*, which specifically binds and biotinylates acetyl-CoA carboxylase (775). A mutated version of BirA (R188G) reverses this specificity, generating a cloud of biotinylation that modifies all proteins close to the enzyme (776). Fusion of the mutated BirA to a protein of interest permits biotinylation of proteins in close proximity to the fusion protein. Biotinylated proteins are then affinity-isolated using streptavidin coupled beads and analyzed by mass spectrometry (777). Recently, D'Costa *et al*. used the BioID system to map interaction networks for the *Salmonella* effectors SifA, PipB2, SseF, SseG, and SopD2 (281). Additionally, several other groups have used this approach to identify novel interactions for mammalian E3 ligases (778–780). I would modify the BioID system to stably express either wild type or catalytically dead SspH1-BirA fusion proteins in U937 and HT29 cells (Figure 6.4). To avoid the loss of potential substrates or protein interactions, I would block substrate processing by inhibiting either the proteasome (MG132) or caspase activation (IDN-6556). The interaction profiles identified through these experiments would be compared to a BirA alone control to rule
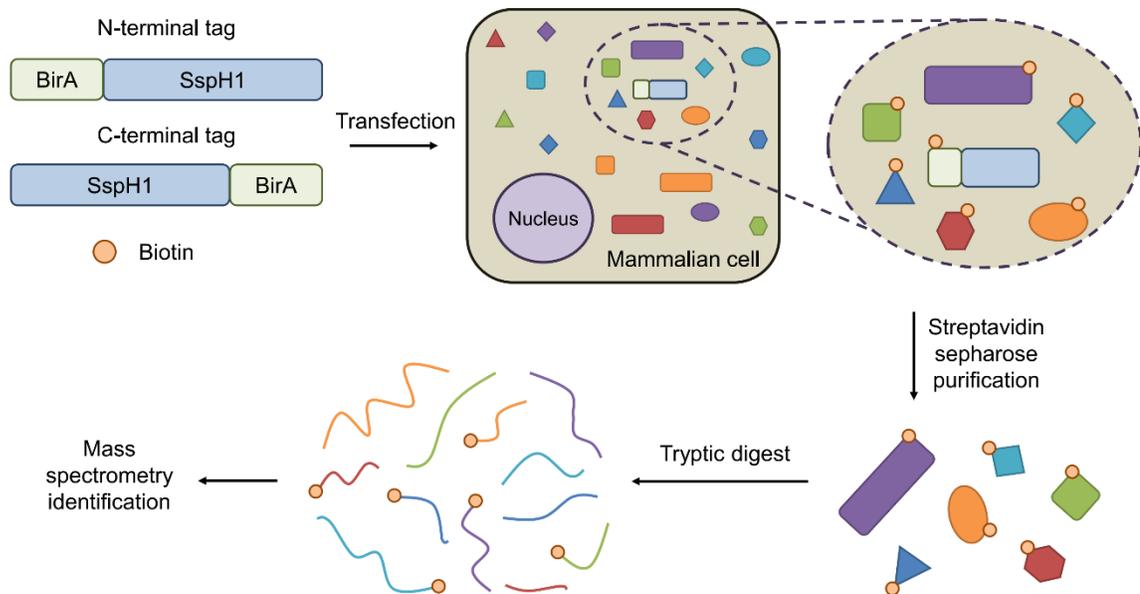
Figure 6.4: BioID strategy to identify SspH1 targets.
A mutated version of BirA (R188G) is fused to either the C-terminus or N-terminus of SspH1 to ensure E3 ligase activity remains unaffected. The functional fusion protein is expressed in mammalian cells (HT29 or U937 cells) in the presence of biotin. BirA biotinylates host proteins in close proximity to the fusion protein. All biotinylated proteins are isolated using streptavidin sepharose purification. Isolated proteins are subjected to tryptic digestion and analyzed via mass spectrometry to identify the interaction map of SspH1. Figure adapted from Sears *et al.*, 2019 (777).

out any potential artifacts. Unlike the UBAIT system, I would expect the BioID approach to provide a proximity-based interaction profile of SspH1 that may contain novel host substrates.

The third approach involves quantitative ubiquitination site profiling using diGly proteomics. This approach uses antibody-based enrichment of proteins containing a diagnostic Gly-Gly dipeptide that is generated from tryptic digestion of ubiquitinated proteins (781). The discriminatory power of the proteomics screen for diGly-remnant containing proteins is enhanced further through combination with stable isotope labelling by amino acids in cell culture (SILAC). In this system, heavy lysine is added to cell culture media to be incorporated in translation of potential E3 ligase substrates (782). Lysates are then harvested from cells subjected to contrasting E3 ligase activity, processed via tryptic digestion and diGly immunoaffinity-based enrichment, and analyzed by mass spectrometry (781). Fiskin *et al.* recently performed this ubiquitinome analysis in response to *Salmonella* infection but used the laboratory strain SL1344, which lacks *sspH1* (783). I would repeat this investigation and instead compare the ubiquitinome between 14028 and Δ*sspH1* infected HT29 and U937 cells with and without MG132 treatment (Figure 6.5). Given the extensive use of the diGly-SILAC system in ubiquitinome analysis (233,784–786), I would expect an in-depth SspH1-related ubiquitination profile from the investigation.

All three substrate screening approaches discussed regarding SspH1 also apply to the SspH1-related protein identified in cluster 3 of section 5 (figure 5.7). However, it is important to determine whether this related protein functions differently from SspH1

A.

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLR**GG**
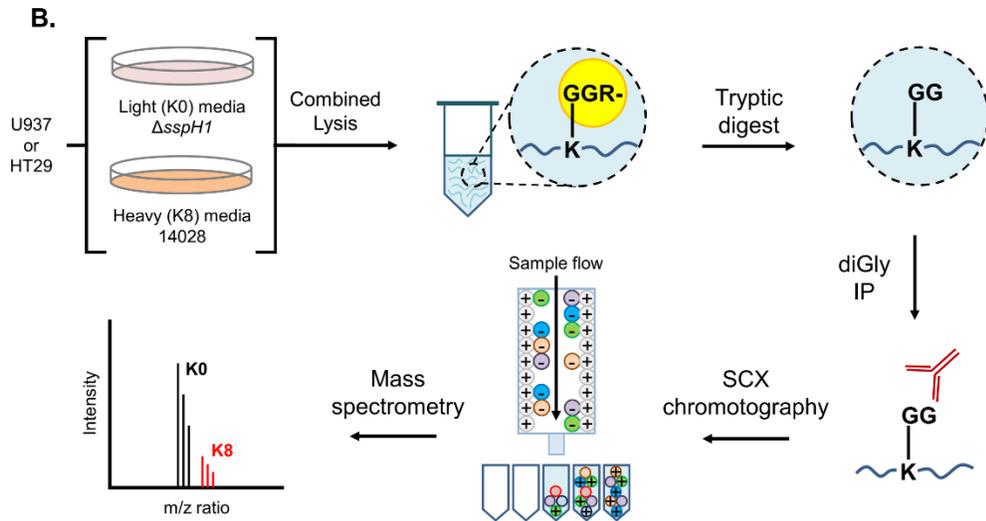
Figure 6.5: Combined SILAC with diGly proteomics strategy to identify SspH1 targets.
(A) DiGly proteomics make use of the two glycines (bold and underlined) at the C-terminus of ubiquitin. (B) This strategy begins with two separate cultures of mammalian cells in light lysine (K0) media and heavy lysine (K8) media. The K0 culture is infected with the Δ*sspH1* mutant and the K8 culture is infected with wild type (14028). Following infection, the lysates are combined and are subjected to tryptic digest. Digestion with trypsin removes ubiquitin from ubiquitinated proteins and leaves a diGly remnant attached to the formerly ubiquitinated lysine residue. Proteins with this diGly remnant are isolated following digestion via diGly immunoprecipitation (IP). Immunoprecipitated proteins are further purified through strong cation exchange (SCX) chromatography. The chromatography fractions are analyzed via mass spectrometry where specific proteins levels are compared between both infections using the mass difference in lysine isotopes. Figure adapted from Fulzele and Bennett, 2018 (781).

before screening for potential substrates. I would first examine the NEL and LRR domains between the SspH1-related protein, SspH1, and SspH2 to compare sequence homology, interaction residues, and domain conservation. Secondly, I would generate a fluorescently tagged version of the protein and monitor its location within transfected cells as SspH1 localizes to the nucleus, whereas SspH2 is dispersed along the cell periphery (235). Lastly, I would assess PKN1 degradation via transient transfection of the SspH1-related protein or during *Salmonella* infection with a strain expressing the SspH1 variant. If the results from these experiments distinguish the SspH1-related protein from SspH1, I would then proceed with screening for novel substrates.

6.7. Final Remarks

The genus *Salmonella* is consistently studied to determine what factors contribute to pathogenesis in humans. Despite decades of research and advancements in food safety strategies, *Salmonella* continues to be a major cause of foodborne illness in both developing and developed countries. The work presented in this thesis analyzed *Salmonella* from molecular, cellular, and food safety perspectives to improve our understanding of the many aspects associated with the foodborne pathogen. Through my studies on food recalls, I determined that *Salmonella* is a leading contributor to microbial-related recalls in Canada and is chiefly responsible for recalls involving nuts, edible seeds, and spices. I also discovered that *Salmonella*-related recalls involving produce food products have substantially increased from 2000-2009 to 2010-2017. From my work with the Syst-OMICS project, I developed an *Acanthamoeba* infection model to screen over one hundred *Salmonella* isolates for their ability to internalize into amoeba. The results of this screening correlated well with the screenings performed by collaborators in

murine and macrophages models, highlighting the utility of Acanthamoeba as an effective screening system to assess *Salmonella* virulence. From my molecular research involving *Salmonella*, I demonstrated that the E3 ubiquitin ligase SspH1 facilitates degradation of PKN1 during *Salmonella* infection in macrophages. While I was unable to determine the impact of SspH1-dependent degradation of PKN1 on pathogenesis, I identified a SspH1-related *Salmonella* protein that is distinct different from SspH1 and SspH2 based on amino acid similarity. Investigating this new SspH1-related protein may provide insight into the contributions of NEL-type E3 ubiquitin ligases during *Salmonella* infection. Altogether, my analysis of *Salmonella* from various perspectives provides evidence to inform future *Salmonella* food safety mitigation strategies, demonstrates the value of Acanthamoeba as a *Salmonella* infection model, and confirms the function of SspH1 during *Salmonella* infection.

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Appendix A

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Herod A, Goodridge L, Rohde J. 2019. Recalls of Foods due to Microbial Contamination Classified by the Canadian Food Inspection Agency, 2000 to 2017. J Food Prot 82:1901–1908.