Dean&Francis

ISSN 2959-409X

Structural Analysis of Salmonella Type III Secretion System and Research Progress on Its Inhibitors

Zichen Song[1],

Leiyin Tang[2]

[1]College of Animal Science and Technology, School of Veterinary Medicine, Zhejiang Agriculture and Forestry University, Hangzhou, Zhejiang, 311399, China
[2]Agricultural and Rural Bureau of Cangnan County, Wenzhou, Zhejiang, 325800, China
[1] E-mail: 2024808041015@stu.zafu.edu.cn
[2] E-mail: leiyintang@163.com

Abstract:

Background: Salmonella is the second most prevalent foodborne pathogen globally, with multidrug resistance increasing by 1.84%-2.69% annually in China, necessitating non-antibiotic therapies. This review aims to analyze the structure and regulatory mechanisms of "Salmonella" Type III Secretion System (T3SS) and advance inhibitor development. We summarize structural insights into T3SS components (basal body, needle complex, transmembrane pore) encoded by SPI-1/SPI-2 pathogenicity islands, and the HilC-RtsA-HilD regulatory network controlling invasion. Key inhibitors demonstrate multi-mechanistic efficacy: salicylidene hydrazides (e.g., INP0007) block effector translocation; Camino side A specifically inhibits T3SS secretion (IC_{50}=20 μM); flavonoids (quercetin/fisetin) suppress SPI-1 genes (hilA, sopA) and disrupt HilD regulator activity, reducing bacterial colonization in vivo by >50%. ATPase SctN/InvC is identified as a novel target.

Conclusion:

T3SS inhibitors exhibit potent anti-virulence effects with low resistance development, providing promising alternatives to conventional antibiotics.

Keywords: Type III Secretion System (T3SS), Salmonella Pathogenesis, T3SS Inhibitors, SPI-1 Regulation

1. Introduction

Prevalence of Salmonella: Salmonella is the second most prevalent foodborne pathogenic microorganism globally, and the bacterial food poisoning incidents it causes often rank first in statistical data from various countries. In China, Salmonella infections account for 70%-80% of foodborne disease incidents caused by bacteria[2]. Prominent Salmonella serotypes comprise Salmonella Typhimurium and Salmonella Enteritidis. In China, Salmonella Typhimurium represents one of the most prevalent serotypes in Salmonella infections.

Antimicrobial resistance issue: Salmonella is exhibiting increasing resistance to multiple antibiotics[3] .Research indicates that between 2006 and 2019 [4],

the number of antimicrobial resistance genes in Salmonella in China significantly increased, with the average number of resistance genes carried by human-derived and non-human-derived non-typhoidal Salmonella genomes increasing by 1.84%and 2.69%, respectively. Addressing the antimicrobial resistance problem of Salmonella can be achieved through non-antibiotic therapies to reduce its pathogenicity and antibiotic dependence[5].

2.The structure of Salmonella pathogenicity islands and type III secretion systems Regulatory network

During the progression of Salmonella infection within the host, numerous genes contributing to the pathogenicity of Salmonella enterica serovar Typhimurium have been identified, notably Pathogenicity Island 1 and 2 (SPI-1 and SPI-2)[6]. The virulence determinants encoded by SPI-1 are essential for bacterial invasion of epithelial cells, whereas SPI-2 genes are transcriptionally activated within the intracellular niche, enabling S. Typhimurium to establish persistence within eukaryotic cells [7]. Although genetic variation exists between SPI-1 and SPI-2, both genomic islands encompass four principal operon classes: 1) structural genes encoding the Type 3 Secretion System (T3SS), 2) regulatory genes, 3) effector protein genes, and 4) chaperone protein genes[8]. The Type III Secretion System (T3SS) represents a sophisticated multiprotein complex[9] widely distributed among Gram-negative bacterial pathogens. This molecular machinery enables direct injection of bacterial effector proteins into eukaryotic host cells, subsequently manipulating cellular signaling cascades to facilitate microbial colonization and drive pathogenic progression. Consequently, the development of T3SS inhibitors represents a promising strategy for targeted therapeutic intervention against pathogenic bacteria.

2.1 The Type III Secretion System (T3SS) of Salmonella represents a sophisticated molecular apparatus comprised of multiple substructures, primarily the basal body, needle complex, and transmembrane pore.

Basal Body

The basal body constitutes a core structural element of the T3SS, spanning the bacterial inner and outer membranes and composed of multiple integral membrane proteins [10]. These proteins assemble into concentric ring structures, providing essential structural integrity for the entire apparatus [11]. For instance, PrgH and PrgK proteins form the inner membrane ring, while InvG assembles into the outer membrane ring . Furthermore, the basal body incorporates an export apparatus comprising proteins including InvA, InvC, and the SpaO-P-Q-R-S complex that collectively mediate effector protein secretion through coordinated molecular interactions.

Needle Structure

The needle structure represents another critical T3SS component, projecting from the bacterial surface and directly mediating effector protein translocation into host cells . This structure is primarily assembled from PrgI and PrgJ proteins. PrgI serves as the major needle subunit, while PrgJ plays a crucial role in needle assembly and effector secretion . The needle typically exhibits a length of 20–150 nanometers and incorporates a tip complex at its distal end, which functions in host cell sensing [13].

Transmembrane Pore

The transmembrane pore is formed by proteins including SipB, SipC, and SipD. This complex creates a conduit within the host cell plasma membrane, permitting the entry of effector proteins into the host cell cytosol. Pore formation is essential for the effective delivery of effector proteins.[14].

Salmonella possesses two distinct T3SSs, encoded by SPI-1 and SPI-2, respectively . The SPI-1-encoded T3SS is activated upon bacterial contact with the host cell membrane. It mediates the delivery of effector proteins into the host cytosol to modulate host cell cytoskeletal dynamics and facilitate bacterial internalization. Conversely, the SPI-2-encoded T3SS is induced within the Salmonella-Containing Vacuole (SCV) following bacterial invasion. The secretory machinery facilitates the directional transport of effector proteins from this membrane-bound compartment into the cytoplasmic matrix of host cells[16]. Although these two T3SSs share functional similarities, they exhibit distinct structural features and secrete different repertoires of effector proteins.

Structural elucidation and functional characterization of the T3SS provide fundamental insights for investigating bacterial pathogenesis and developing targeted antimicrobial agents. The needle-like architecture of the T3SS strongly implies a direct injection mechanism, whereby effector proteins are delivered into the host cytosol, with the needle filament itself acting as a conduit for these partially unfoldedmolecules.

ISSN 2959-409X

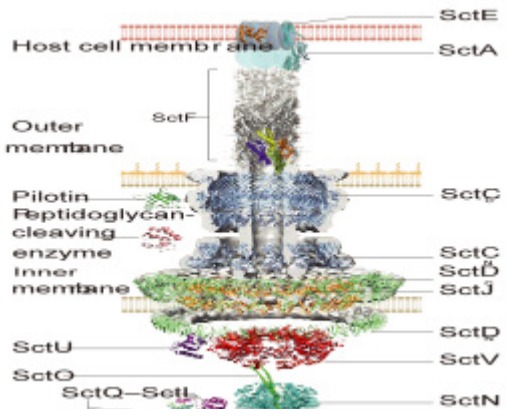

Figure 1 The type III secretion system (T3SS) of Salmonella is composed of the following structures

2.2 Regulatory Mechanisms Governing Salmonella Typhimurium Invasion via T3SS

The invasion mechanism of Salmonella Typhimurium into host cells mediated by the Type III Secretion System (T3SS) is modulated by diverse regulatory genes and environmental factors.

The core regulatory circuitry comprises the HilC-RtsA-HilD feedforward loop, wherein each transcriptional regulator autonomously activates the expression of hilC, rtsA, hilD, and hilA, forming an intricate feedforward network that orchestrates SPI-1 gene expression. HilC, RtsA, and HilD constitute a feedforward regulatory pathway that collectively governs SPI-1 expression. Within this framework, HilD functions as the primary activator, capable of directly stimulating its own transcription and augmenting HilA production through a positive feedback loop.

As members of the AraC/XylS family, HilC and HilD independently counteract the repressive effects of inhibitory elements on the hilA promoter, thereby potentiating HilA expression. Additionally, HilC and HilD directly activate an alternative promoter of the invF operon through a mechanism independent of HilA .[8]

SPI-1 gene expression is regulated by multiple environmental cues, including pH, oxygen tension, osmolarity, bile salts, Mg^{2+} concentration, and short-chain fatty acids. These factors modulate SPI-1 expression by altering the activity of HilC, RtsA, and HilD .

HilA serves as the master regulator of SPI-1 gene expression, binding to promoter regions upstream of prgH and invF to initiate transcription of T3SS structural genes and effector protein genes. HilA expression is co-regulated by HilD, HilC, and RtsA via a complex feedforward mechanism, ensuring SPI-1 genes are expressed under appropriate environmental conditions .

HilD is essential for HilA activation, providing sufficient stimulatory signals to drive HilA expression despite the presence of repressive factors . HilC amplifies HilA expression by alleviating repression mediated by these inhibitory elements. RtsA also plays a critical role in this regulatory network, cooperating with HilC and HilD to collectively control SPI-1 expression .

The physiological implications of this regulatory network are as follows:

By precisely regulating SPI-1 gene expression, Salmonella Typhimurium efficiently colonizes the host intestinal tract and invades host cells. This regulatory system enables bacterial adaptation of virulence potential in response to varying environmental contexts, thereby enhancing persistence and dissemination within the host .

SPI-1 gene expression is further intricately linked to bacterial responses to host immune defenses . By modulating T3SS activity, Salmonella Typhimurium evades host immune surveillance, facilitating prolonged intracellular survival .

The HilC-RtsA-HilD feedforward regulatory network constitutes the central mechanism directing SPI-1 expression in Salmonella Typhimurium . This system integrates multiple environmental signals and regulatory factors to ensure spatiotemporally appropriate expression of SPI-1 genes, thereby promoting bacterial pathogenicity and host adaptation .

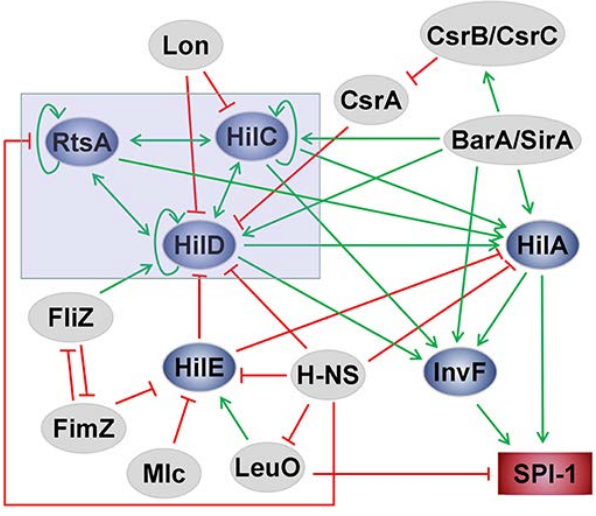

Figure 2 The regulatory network of the type III secretion system (T3SS) in Salmonella

3.Recent Progress in Gram-negative bacteriaT3SS Inhibitors

In recent years, substantial advancements have been achieved in the development of inhibitors targeting the Type III Secretion System (T3SS). Certain inhibitors

function by binding to structural components of the T3SS apparatus, thereby impeding the secretion of effector proteins. Others suppress T3SS expression through interaction with its regulatory factors, while additional compounds disrupt T3SS-associated signal transduction pathways. Consequently, the development of such inhibitors represents a critical strategy for enhancing antibiotic therapeutic efficacy, mitigating the progression of bacterial resistance, providing novel therapeutic modalities, and facilitating the discovery of new antimicrobial agents.

3.1 Salicylidene hydrazide derivatives exhibit broad-spectrum inhibitory activity against the T3SS of diverse Gram-negative pathogens.

For instance, the compound INP0007 interferes with the translocation of Yop virulence proteins, thereby inhibiting Yersinia invasion of HeLa cells without affecting host cell viability [36]. Kauppi et al. [28] employed a luciferase reporter gene assay in live Yersinia pseudotuberculosis to screen salicylidene hydrazide derivatives, including INP0007, from a library of 9,400 compounds. These derivatives potently suppressed reporter gene signals driven by the yopE promoter and the secretion of effector proteins at low micromolar concentrations, without impacting bacterial proliferation. INP0007 specifically blocks T3SS-mediated secretion in Yersinia, preventing bacterial invasion of HeLa cells . Furthermore, in uninfected HeLa cells cultured in the presence of INP0007 (50 μM), the compound significantly attenuated YopE-induced cytotoxicity, demonstrating its capacity to inhibit the translocation of multiple Yop effector proteins.

3.2

The natural glycolipid caminoside A, isolated from marine sponges, inhibits the secretion of the effector protein EspB via the enteropathogenic Escherichia coli (EPEC) T3SS. Caminoside A is a glycolipid compound purified from the sponge Caminus sphaeroconia, collected from the upper walls of the Toucari Cave in Dominica. This sponge inhabits a specific ecological niche, and bioactivity-guided screening identified its extracts as possessing potent T3SS inhibitory properties. Caminoside A effectively inhibits the EPEC T3SS, significantly suppressing EspB secretion without affecting EspC secretion. As EspC can also be secreted via the type IV secretion system, this finding indicates that caminoside A specifically targets the T3SS. Furthermore, caminoside A exhibits no inhibitory effect on the growth of Gram-negative bacteria such as E. coli (MIC >100 μg/mL), yet demonstrates antibacterial activity against Gram-positive pathogens including methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant Enterococcus (VRE) (MIC = 12 μg/mL for both). The half-maximal inhibitory concentration (IC_{50}) of caminoside A for T3SS inhibition is 20 μM, signifying its potency at relatively low concentrations. This inhibition attenuates EPEC pathogenicity without bactericidal effects, providing a promising avenue for developing novel antimicrobial agents, particularly in light of escalating antibiotic resistance. Although caminoside A demonstrates significant T3SS inhibitory potential, its further development and application are hindered by its complex structure and synthetic challenges. Nonetheless, its discovery provides an important reference point for exploring novel antimicrobial compounds derived from marine organisms. Collectively, these advancements have furnished new integrated concepts and leads for T3SS inhibitor research, with the development of specific inhibitors representing a significant milestone for future studies. The research objective encompasses a thorough investigation into the mechanism of inhibitors targeting the Salmonella T3SS and the exploration of their potential clinical applications. Currently, beyond the T3SS inhibitors documented in the literature, several inhibitors are under development and undergoing clinical trials.

4.Analyzing the invasion and virulence mechanisms of Salmonella

4.1 Invasion process dependent on SPI-1 T3SS

The SipA protein of Salmonella is encoded by the SPI-1 T3SS and is a dual-function protein . Its C-terminus can bind and stabilize F-actin while enhancing the activity of T-plastin, leading to membrane invagination and promoting bacterial entry into host cells [30]. Meanwhile, its N-terminus contains a functional domain that activates the signal transduction pathway in epithelial cells, which facilitates the transendothelial migration of neutrophils

4.2 Salmonella Effector Protein-Mediated Formation of SCV and Intracellular Survival

After entering host cells, Salmonella forms a membrane-bound vesicular structure in the cytoplasm called the Salmonella-containing vacuole (SCV) . During early SCV formation, the T3SS-1 effector protein SopB exerts phosphoinositide phosphatase activity to recruit the host cell's Rab5 to the phagosomal membrane. This stimulates the generation of PI(3)P (phosphatidylinositol 3-phosphate) and activates the Akt kinase signaling cascade, while promoting LAMP-1 translocation to the SCV [9], thereby facilitating bacterial survival within host cells.

ISSN 2959-409X

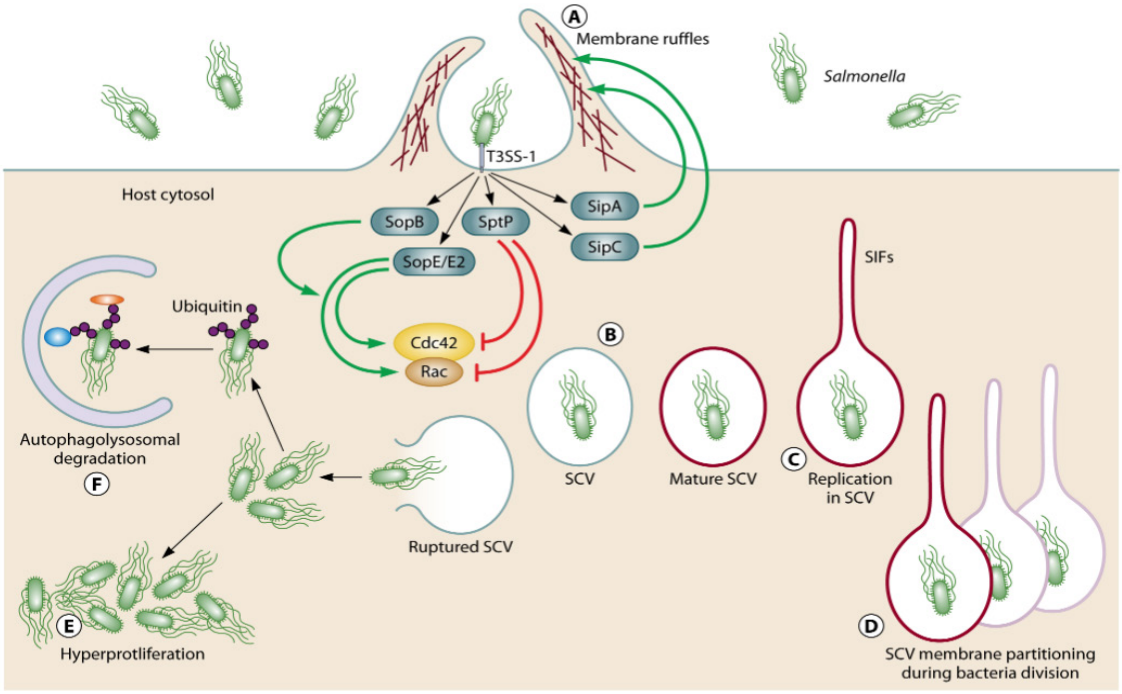

Figure 3 The strategies of Salmonella invading host intestinal cells

The virulence of Salmonella is closely associated with multiple pathogenicity islands (SPIs), particularly SPI-1 and SPI-2. The T3SS-related effector proteins encoded by SPI-1 are primarily involved in Salmonella invasion and translocation, facilitating bacterial entry into host cells, whereas the SPI-2-encoded T3SS primarily governs Salmonella's intracellular persistence while concurrently facilitating systemic dissemination within the host organism. These effector proteins suppress host immune responses by modulating host cell signal transduction, cytokine secretion, and other processes, thereby enhancing Salmonella's intracellular survival capacity and contributing to the maintenance of persistent infection.

5. Inhibitors targeting Salmonella T3SS through different mechanisms

5.1 Suppression of T3SS-1 Genes

This suppressive effect may be mediated through modulation of cAMP levels or cAMP receptor protein (CRP) activity. The cAMP-CRP complex stimulates expression of the ribosome-associated inhibitor A (YfiA), encoded by yfiA, which contributes to maintaining the stability of HilD, a principal transcriptional regulator of T3SS-1. HilD serves as the master regulator of Salmonella pathogenicity island 1 (SPI-1) gene expression, and its stability is critical for T3SS-1 functionality. By inhibiting cAMP-CRP complex activity, L-arabinose indirectly reduces YfiA expression, leading to decreased HilD stability and ultimately suppressing T3SS-1 gene expression.

Studies indicate that L-arabinose supplementation enhances the initial intestinal colonization of Salmonella enterica serovar Typhimurium in antibiotic-pretreated mice. This effect may arise from L-arabinose-mediated suppression of T3SS-1 expression, potentially enhancing bacterial adaptability to the intestinal environment. Although T3SS-1 suppression may attenuate bacterial virulence, thisinhibition could facilitate evasion of host immune surveillance within the intestine, thereby promoting enhanced colonization.Despite the suppression of T3SS-1 expression by L-arabinose, this inhibitory effect may be counterbalanced by other bacterial mechanisms in vivo. For example, the bacterium may activate alternative virulence factors or adaptive responses to sustain pathogenicity. Furthermore, L-arabinose may indirectly promote bacterial survival and dissemination through modulation of host immune responses.

5.2 Suppression of Host Cell Invasion by Quercetin

Quercetin inhibits Salmonella enterica serovar Typhimurium invasion of host cells and ameliorates Salmonella-mediated host cell damage.β-Galactosidase activity assays and Western blot analysis demonstrated that quercetin significantly inhibits expression of SPI-1 genes (hilA,

sopA) and effector proteins (SipA, SipC) . These findings suggest quercetin directly impairs the function of the Salmonella Typhimurium type III secretion system (T3SS), consequently inhibiting its invasive capacity. In vivo experiments revealed that quercetin significantly reduces bacterial colonization in infected mice and alleviates cecal pathology, indicating efficacy not only in vitro but also in ameliorating infection symptoms in vivo, highlighting its therapeutic potential. As a small-molecule T3SS inhibitor, quercetin represents a potential alternative to conventional antibiotics for treating Salmonella Typhimurium infections. Being a natural plant flavonoid with favorable biocompatibility and low toxicity, its inhibitory effect on the Salmonella T3SS positions quercetin as a promising novel antibacterial agent.[14]

5.3 Suppression of Salmonella Invasion by Fisetin Targeting SPI-1 Regulation

Fisetin suppresses Salmonella invasion of host cells by targeting the SPI-1 regulatory network. Specifically, fisetin inhibits HilD, a key transcriptional regulator of SPI-1, thereby disrupting its interaction with target gene promoters . HilD activity is essential for the expression of T3SS-1 effectors and structural components. By impairing HilD function, fisetin significantly reduces SPI-1 gene expression, consequently diminishing T3SS-1 activity. Fisetin treatment disrupts the interaction between HilD and its target gene promoters, inhibiting the expression of T3SS-1-associated effectors and structural proteins. This suppression occurs via modulation of HilD activity rather than direct action on effector or structural genes, indicating fisetin inhibits T3SS-1 functionality through regulation of the SPI-1 transcriptional cascade.[13]

In vivo results demonstrate that fisetin significantly reduces bacterial colonization in infected mice and mitigates cecal pathological damage. This indicates fisetin not only inhibits Salmonella invasion in vitro but also effectively reduces the inflammatory response and tissue damage caused by the pathogen in vivo.

These inhibitors can treat various inflammations caused by Salmonella and exhibit low resistance, indicating their potential importance in future bacterial therapies.

5.4 Inhibiting ATPase activity: By suppressing the ATPase of T3SS (such as SctN1), the secretion of effector proteins can be blocked.

Currently, no highly effective inhibitors specifically targeting the SctN1 protein of the T3SS have been identified. In Salmonella, SctN1 serves as the ATPase component of the Type III Secretion System (T3SS) [49]. Its primary functions include: acting as a molecular motor to provide the energy required for protein export (likely), and being essential for the formation of the Type III Secretion Apparatus (T3SS), proper protein secretion, host cell invasion, and virulence [50]. The SctN1 protein, also known as the InvC gene, could serve as a potential direction for future research on T3SS inhibitors.

6. Conclusion

Targeting the virulence mechanisms of Salmonella, particularly the Type III Secretion System (T3SS), represents a promising therapeutic strategy. In summary, inhibitors targeting the T3SS overcome drug resistance through three pathways:1) Bypassing traditional antibiotic targets (e.g., quercetin acting on HilD protein),2) Reducing selective pressure (due to the non-bactericidal nature of caminoside A),3) Blocking virulence rather than growth (as demonstrated by INP0007).While L-arabinose suppresses T3SS-1 expression, potentially aiding immune evasion and colonization, compensatory host mechanisms may sustain pathogenicity. More effectively, natural compounds like quercetin and fisetin directly inhibit T3SS functionality: quercetin suppresses SPI-1 gene (hilA, sopA) and effector (SipA, SipC) expression, while fisetin targets the key regulator HilD, disrupting the SPI-1 transcriptional network and T3SS-1 activity. Both compounds significantly reduce bacterial invasionin vitroand mitigate infection, colonization, and tissue damagein vivo. Additionally, inhibiting essential ATPase components like SctN1 (InvC) offers a potential future avenue for blocking effector secretion. Collectively, these T3SS inhibitors demonstrate significant potential for treating Salmonella-induced inflammation with the advantage of low resistance development, highlighting their importance for future antibacterial therapies.

References

[1] Lostroh, C. C. (2001). Activation of the invF and prgH promoters by HilA, a Salmonella virulence factor. Harvard University.

[2] Saleh, D. O., Horstmann, J. A., Giralt-Zúñiga, M., Weber, W., Kaganovitch, E., Durairaj, A. C., ... & Erhardt, M. (2023). SPI-1 virulence gene expression modulates motility of Salmonella Typhimurium in a proton motive force-and adhesins-dependent manner. PLoS pathogens, 19(6), e1011451.

[3] Zhu, Y., Lai, H., Zou, L., Yin, S., Wang, C., Han, X., ... & Liu, S. (2017). Antimicrobial resistance and resistance genes in Salmonella strains isolated from broiler chickens along the slaughtering process in China. International journal of food microbiology, 259, 43-51.

[4] Kumar, M., Sarma, D. K., Shubham, S., Kumawat, M., Verma, V., Nina, P. B., ... & Tiwari, R. R. (2021). Futuristic

ISSN 2959-409X

non-antibiotic therapies to combat antibiotic resistance: A review. Frontiers in microbiology, 12, 609459.

[5] Gerlach, R. G., & Hensel, M. (2007). Salmonella pathogenicity islands in host specificity, host pathogen-interactions and antibiotics resistance of Salmonella enterica. Berliner und Munchener tierarztliche Wochenschrift, 120(7/8), 317.

[6] Ishizuka, H., Hanamura, A., Inada, T., & Aiba, H. (1994). Mechanism of the down-regulation of cAMP receptor protein by glucose in Escherichia coli: role of autoregulation of the crp gene. The EMBO journal, 13(13), 3077-3082.

[7] Notti, R. Q., & Stebbins, C. E. (2016). The structure and function of type III secretion systems. Virulence mechanisms of bacterial pathogens, 241-264.

[8] Gerlach, R. G., & Hensel, M. (2007). Salmonella pathogenicity islands in host specificity, host pathogen-interactions and antibiotics resistance of Salmonella enterica. Berliner und Munchener tierarztliche Wochenschrift, 120(7/8), 317.

[9] Fàbrega, A., & Vila, J. (2013). Salmonella enterica serovar Typhimurium skills to succeed in the host: virulence and regulation. Clinical microbiology reviews, 26(2), 308-341.

[10] Lerminiaux, N. A., MacKenzie, K. D., & Cameron, A. D. (2020). Salmonella pathogenicity island 1 (SPI-1): the evolution and stabilization of a core genomic type three secretion system. Microorganisms, 8(4), 576.

[11] McGhie, E. J., Brawn, L. C., Hume, P. J., Humphreys, D., & Koronakis, V. (2009). Salmonella takes control: effector-driven manipulation of the host. Current opinion in microbiology, 12(1), 117-124.

[12] Mallo, G. V., Espina, M., Smith, A. C., Terebiznik, M. R., Alemán, A., Finlay, B. B., ... & Brumell, J. H. (2008). SopB promotes phosphatidylinositol 3-phosphate formation on Salmonella vacuoles by recruiting Rab5 and Vps34. The Journal of cell biology, 182(4), 741-752.

[13] Agbor, T. A., & McCormick, B. A. (2011). Salmonella effectors: important players modulating host cell function during infection. Cellular microbiology, 13(12), 1858-1869.

[14] Li, Q., Wang, L., Liu, S., Xu, J., Song, Z., Chen, T., ... & Lv, Q. (2021). Inhibition of Salmonella enterica serovar Typhimurium Type III Secretion System and Infection Using Small Molecule Quercitrin.