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# Bacterial Type VI Secretion System (T6SS) Driven Microbial Warfare for Improving Gut Health

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## Abstract

*Campylobacter jejuni (C. jejuni)* is a leading cause of foodborne diseases, with poultry serving as a major source of transmission. Like other Gram-negative pathogens, *C. jejuni* employs diverse toxin secretion systems, including the recently characterized Type VI Secretion System (T6SS), to facilitate pathogenesis, self-survival, and interspecies competition in the gut environment. Our previous findings demonstrated that *C. jejuni* T6SS functionality can induce "self-depletion" when interacting with appropriate target cells, such as *E. coli*, particularly under environmental stress. We hypothesized that exploiting T6SS-driven "predation cost" could improve gut health by employing selective targets or interacting bacteria. Using *in vitro* co-culture assays with the probiotic *Lactobacillus johnsonii (L. johnsonii)*, revealed significant growth inhibition and membrane damage in T6SS-positive *C. jejuni* compared to T6SS-negative strains. This differential response was associated with a modest increase in the release of hemolysin co-regulated protein (Hcp), a hallmark of T6SS effector. Scanning electron microscopy further revealed surface damage consistent with bacteriocin-mediated stress. We propose that the activation of T6SS may have some role in sensitizing *C. jejuni* to anti-microbial metabolites or bacteriocins produced by *L. johnsonii*, enhancing the vulnerability of the bacteria. These findings underscore the potential of manipulating microbial competition and T6SS dynamics as a novel strategy for gut microbiota modulation and pathogen control.

**Keywords:** Bacterial Type VI secretion systems (T6SS), *Campylobacter jejuni*, *Lactobacillus johnsonii*, Inter-microbial interaction, Gut health

## Introduction:

Pathogenic bacteria have evolved mechanisms to thrive in adverse environments and secure competitive advantages. These mechanisms often involve specialized secretion systems, which enable the export of toxins or the injection of effector proteins into neighboring cells (Pickard et al., 2017). Recently, it was found that some *Campylobacter jejuni* (*C. jejuni)* strains possess specialized secretion machinery, Type-VI secretion system (T6SS), which acts as an inverted bacteriophage to deliver effector proteins into target cells in a contact-dependent manner (Lertpiriyapong et al., 2012). T6SS is present in ~25 % of Gram-negative bacteria, including other pathogens such as *Pseudomonas aeruginosa* (Mougous et al., 2006), *Vibrio cholerae* (Nag et al., 2018), *Burkholderia thailandensis* (Si et al., 2017), *Yersinia pseudotuberculosis* (Wang et al., 2015) and *Campylobacter jejuni* (Gupta et al., 2021). Our group recently demonstrated that *C. jejuni* T6SS plays a critical role in exhibiting distinct phenotypic and functional changes under altered growth conditions in the presence of prey bacteria. This observation suggests a dual functionality of the T6SS, involving both the import and export of toxins and other macromolecules (Gupta et al., 2024).

Given the diversity in microbial populations within the gut, our previous work raises the possibility of leveraging the target-driven functionality of T6SS as a novel approach for precisely targeting specific pathogens within the gut without compromising the commensal microbial community. Conventional strategies, such as dietary supplementation, fecal microbiota transplantation (FMT), bacteriophage therapy and competitive exclusion show promise but often struggle to selectively target gut pathogens without disrupting commensal microbial populations (Caflisch et al., 2019).

To this end, we hypothesize that the T6SS, while conferring competitive advantages through effector export, may also mediate unintended costs via macromolecule uptake under stress conditions, revealing a "dual role" in both toxin secretion and environmental sensing. This bidirectional activity suggests a regulatory mechanism where T6SS engagement with prey bacteria targets competitors and may facilitate "import" of other biologicals from the extracellular milieu, potentially compromising their cell

viability (Gupta et al., 2024). This mechanistic duality positions T6SS as a potential target for precision microbiome modulation. By harnessing interspecies dynamics and leveraging commensal antagonists such as *Lactobacillus johnsonii*, (*L. johnsonii*), it may be possible to suppress pathogenic *C. jejuni* strains while preserving beneficial microbes selectively.

Based on this hypothesis, the present study investigated the potential for interspecies interactions between *C.jejuni* and the well-characterized probiotic *L. johnsonii*, to determine whether selective targeting of T6SS-positive *C. jejuni* strains could be achieved. The choice of *L. johnsonii* as an interacting partner was driven by its ability to secrete anti-microbial peptides, such as bacteriocins, which confer both self-preservation and a competitive edge in microbial ecosystems (Vazquez-Munoz et al., 2022). Using an optimal condition for co-culturing both bacteria, we showed that in the presence of *L. johnsonii*, a marked reduction in the T6SS-positive *C. jejuni* population could be achieved, in contrast to when the T6SS-negative strain was used as a competing bacterium.

Collectively, this study highlights some antagonizing functionality of T6SS during interspecies interactions. By exploiting prey-driven T6SS mechanisms, we propose further exploring these unique attributes of T6SS, which can help formulate novel dietary formulations for improving gut health in poultry or other susceptible hosts.

## Materials and Methods:

### *Bacterial strains and culture medium*

The T6SS-positive strain of *C. jejuni* (18aM), whole genome sequenced isolate (*Campylobacter jejuni* strain 18aM, whole genome shotgun sequencing project, GenBank: JAPCIC000000000.1) and T6SS-negative strains of *C. jejuni* (3A1) were used in this study. *L. johnsonii* Strain 135-1-CHN and HM-643 were obtained from BEI Resources, NIAID, and NIH, USA as part of the Human Microbiome Project.

### *Phenotypic characterization of the T6SS-positive C. jejuni*

Considering hemolytic co-regulated protein (Hcp) is the key effector protein of *C. jejuni* T6SS, we performed PCR to detect the presence of the *hcp* gene in the T6SS as per the conditions published previously (Singh and Mallick, 2019). The specific primer set used for amplifying the *hcp* gene was:

FP: 5’ CAAGCGGTGCATCTACTGAA 3’; RP: 5’ TAAGCTTTGCCCTCTCTCCA 3’

Further, to check the functionality of *C. jejuni* T6SS, we performed a Western blot analysis of the culture supernatant of T6SS-positive (18aM) and negative strains (3A1) of *C. jejuni* as per the method described previously (Gupta et al., 2021). Briefly, cell-free supernatant from *C. jejuni* ($OD_{600}$~ 1.0) was precipitated with 10% TCA at 4 °C, and the protein pellet was washed with ice-chilled acetone, resuspended in PBS, and subjected to SDS-PAGE. Proteins were transferred to a PVDF membrane, blocked with 5% BSA in TBS, and probed with rabbit anti-Hcp primary antibody, followed by HRP-conjugated goat anti-rabbit secondary antibody. Finally the blot was detected using 3,3′-Diaminobenzidine (DAB) dissolved in Tris-buffered saline (TBS).

Furthermore, the functionality of T6SS was checked by the indirect ELISA method using the culture supernatants of T6SS-positive (18aM) and negative (3A1) as per the methods published elsewhere (Gupta et al., 2021).

### *Assessing the anti microbial activity of L. johnsonii against C. jejuni biomass*

Since *L. johnsonii* is known to secrete bacterial metabolites, including bacteriocins, which have an antibacterial role, we checked the direct probiotic effect of *L. johnsonii* against biofilm (disruption) formed by *C. jejuni* (Vazquez-Munoz et al., 2022).

### *Biofilm disruption assay*

### *Crystal violet staining of biofilm*

The effect of the probiotic activity of *L. johnsonii* was examined for biofilm disruption (formed by *C. jejuni*) as per published methods (Singh et al., 2019). Briefly, the culture supernatant of *L. johnsonii* was prepared as mentioned in the earlier section. Biofilm formed by *C. jejuni* was collected and treated with *L. johnsonii* culture supernatant for 24 h at microaerophilic conditions. The *C. jejuni* biofilm treated with only medium was kept as a negative control. After incubation, the biofilm was washed with 1× PBS, followed by staining with crystal violet (0.5 %, v/v) for 20 min. Following, the biofilm was washed with 1× PBS to remove unbound dye and then air dried. Next, 30 % glacial acetic acid (v/v) was added to dissolve the stain and further quantified using a microplate reader at 595 nm (BioTek). The experiment was performed in triplicate (n=3) under similar conditions.

### *Electron microscopy (FESEM) of biofilm*

The FESEM was carried out at IISER, Kolkatato visualize the biofilm degradation by the cell-free culture supernatant of *L. johnsonii*. Briefly, *C. jejuni* biofilm grown on glass coverslips was treated with the culture supernatant of *L. johnsonii* as described in the previous

section. Next the slides were processed for FESEM as per the method described earlier (Biswas et al., 2024).

***Confocal imaging of biofilm***

To further assess the ability of *L. johnsonii* in biofilm disruption, cell-free culture supernatants were used to treat the biofilm formed by *C. jejuni* on a coverslip as described earlier (Singh et al., 2019). Acridine orange (0.01 %, w/v) was used to stain the biofilm for 2 min to see the changes in the structure of the biofilm. Finally, the coverslips were washed with 1× PBS and mounted on a glass slide using Vecta-shield mounting media (Vector Laboratories, USA). The fluorescent biofilm biomass was captured in a Leica confocal laser scanning microscope using a FITC filter ($\lambda_{ex}$: 488 nm, $\lambda_{em}$: 535 nm) and further processed using Fiji software at IISER, Kolkata.

***Optimizing co-culture conditions for C. jejuni and L. johnsonii***

Following confirmation of the phenotypic and functional characteristics of both bacteria, optimal culture conditions were established to study the effect of inter-bacterial interaction between T6SS-positive or -T6SS-negative strains of *C. jejuni* and *L. johnsonii*.

Briefly, *C. jejuni* ($1\times10^6$ CFU/mL) and *L. johnsonii* ($1\times10^7$ CFU/mL) were mixed in Mueller-Hinton (MH) broth and co-incubated for different periods (5 h- 25 h) at 37˚C in a microaerophilic condition (85% $N_2$,10% $CO_2$ and 5% $O_2$ pressure). After co-incubation, bacterial samples were washed with 1× PBS and further serially diluted using MH broth to spread onto blood-free *Campylobacter* Selective Agar Base medium (HiMedia, India) supplemented with CAT selective supplement (cefoperazone 8 mg/L, amphotericin 10 mg/L, and teicoplanin 4 mg/L) (HiMedia, India). The plates were incubated at 37 ˚C for a duration of 24 h. Finally, characteristic milky white colonies (*C. jejuni*) on the plates were counted (CFU/mL). As controls, *C. jejuni* or *L. johnsonii* were maintained under similar conditions. The experiment was performed in triplicate (n=3).

We also checked their growth profile separately in MRS broth (deManRogosa Sharpe broth, common media for *L. johnsonii*) or MH broth (Mueller-Hinton broth, common media for *C. jejuni*). Their growth profile was monitored with the function of time and temperature.

***Effect of inter-species interaction on C. jejuni morphology and functionality***

To see the effect of co-culture on *C. jejuni* morphology due to inter-bacterial interaction and metabolites/bacteriocins secreted by *L. johnsonii,* Field Emission Scanning Electron Microscopy (FESEM) was performed at IISER, Kolkata. For this, samples of competing bacteria were fixed on glass coverslips using 2.5% v/v glutaraldehyde (in PBS) after 7 h of incubation. Next, the fixed samples were serially dehydrated with 35%, 50%, 70%, 90% and 100% ethanol. The samples in 100% ethanol were vacuum-dried, fixed on aluminum stubs with silver conductive paint, and sputter-coated with gold and electron micrographed (Biswas et al., 2024).

To check whether the co-culture of T6SS-positive *C. jejuni* with *L. johnsonii* has any effect on effector secretion via T6SS, the culture supernatant was subjected to ELISA to test the presence of Hcp. For ELISA, culture supernatant was used as coating antigen dissolved in carbonate-bicarbonate buffer (pH~9.3), followed by blocking with 2-5% BSA. Next, rabbit polyclonal antibody raised against Hcp was used as the primary antibody; while Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) was used as the secondary antibody. Finally, 3,3′,5,5′-Tetramethylbenzidine (TMB) was added as the substrate for HRP to develop color followed by adding 1M $H_2SO_4$ to stop the reaction. The absorbance of each well was measured by a UV-Vis multi-plate reader (BioTek, USA) at 450 nm(Singh et al., 2019).

***Statistical analysis***

The GraphPad Prism software (Version 8.0.1) was used for graphical presentations and data analysis. The Shapiro-Wilk test was performed to confirm the normal distribution. The Student t-test (two-tailed, unpaired) or non-parametric Mann-Whitney U test was performed to compare the significance among various experimental groups. *p value $\leq$ 0.05, **p value $\leq$ 0.01 and ***p value $\leq$ 0.001 were considered as statistically significant.

## Results and Discussion:

***Detection of Hcp in the C. jejuni culture supernatant***

After confirming the presence of a specific amplified gene product at ~463bp (Figure 1A) using hcp-specific primer set, we next verified the functionality of the T6SS of the selected isolate of *C. jejuni*. Since Hemolysin co-regulated protein (Hcp) is considered a key effector protein secreted by a functional T6SS (Lertpiriyapong et al., 2012; Noreen et al., 2018; Singh et al., 2019), Western blot analysis was performed, which showed the corresponding protein at ~21 kDa, confirming the presence of Hcp in the culture supernatant of *C. jejuni* strain 18aM (but not in the negative strain, 3A1) used herein (Figure 1B).

Also, the ELISA data showed the presence of Hcp in 18 aM (T6SS-positive strain) in comparison to the 3A1 strain of *C. jejuni (*T6SS-negative*)* (Figure 1C).

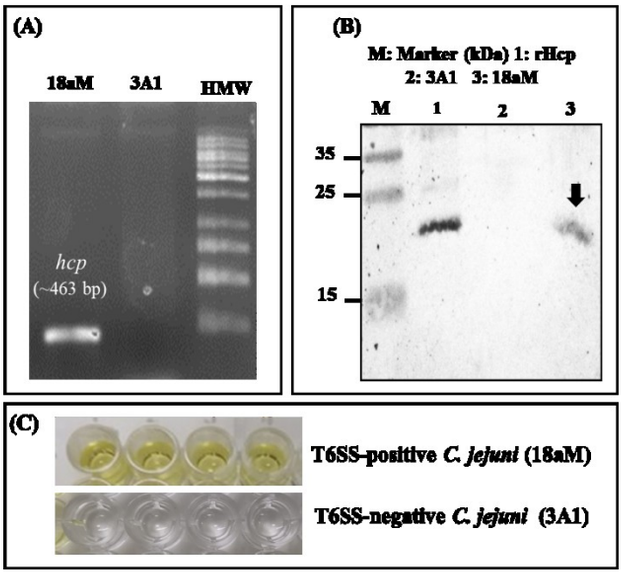


**Figure 1: PCR amplification of the hcp gene of *C. jejuni* showing a specific gene product corresponding to the size of the hcp gene (~463bp) (A) Western Blot analysis of T6SS-positive *C. jejuni* culture supernatant precipitated using TCA. The protein blot (~ 21 kDa) detected in Lane 3 corresponds to the size of the rHcp protein (Lane 1). No such protein could be detected in the T6SS-negative strain (Lane 2; strain 3A1) (B) Detection of Hcp in the culture supernatant by indirect ELISA (C) showing marked color differences between the wells marked for T6SS-positive and -T6SS-negative strains of *C. jejuni*.**

***Culture supernatant of L. johnsonii disrupts biofilm formed by C. jejuni**:*

The antibiofilm activity of *L. johnsonii* secretomes (collected after 48h) present in the culture supernatant was tested against *C. jejuni* biofilm (Aiba et al., 2015). The data/images obtained from crystal violet (CV) and Acridine orange staining as well as FESEM micrographs, indicate noticeable changes suggesting effective disruption of biofilm biomass formed by growing *C. jejuni* under environmental stress (Figure 2A). Moreover, confocal microscopy of acridine orange-stained biomass further indicates patchy morphology of *C. jejuni* biofilm when treated with *L. johnsonii* culture supernatant compared to untreated controls (Figure 2B).

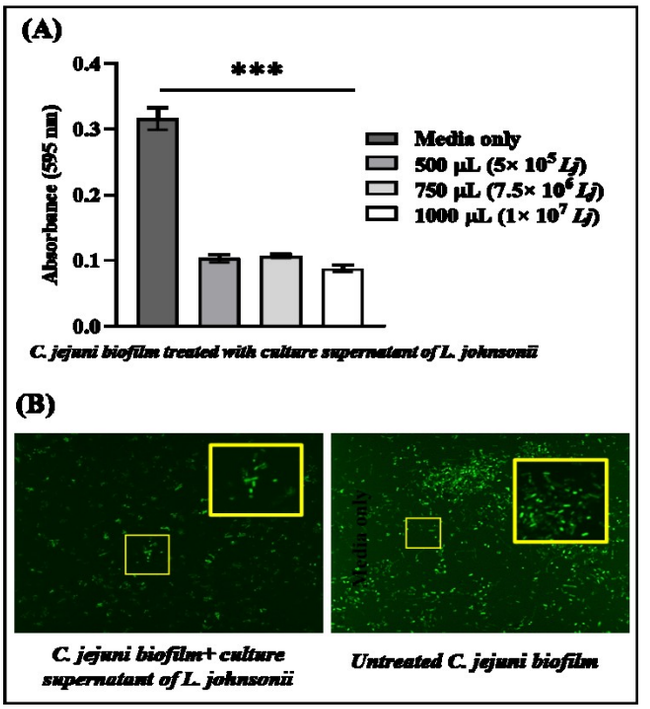


**Figure 2: (A) Biofilm disruption by *L. johnsonii* culture supernatant. Crystal violet assay suggests a marked reduction in biofilm biomass of T6SS-positive *C. jejuni* when treated with the different volumes of the culture supernatant of *L. johnsonii* compared to the untreated control (B) Laser scanning Confocal microscopic images of acridine orange-stained biofilm, indicating a patchy biofilm when treated with *L. johnsonii* culture supernatant compared to untreated biofilms of *C. jejuni* (uniform appearance)**

Comparative image analysis of *C. jejuni* biofilm biomass treated with or without the culture supernatant of *L. johnsonii* captured by FESEM microscopy also marked cellular degradation of *C. jejuni* (Figure 3).

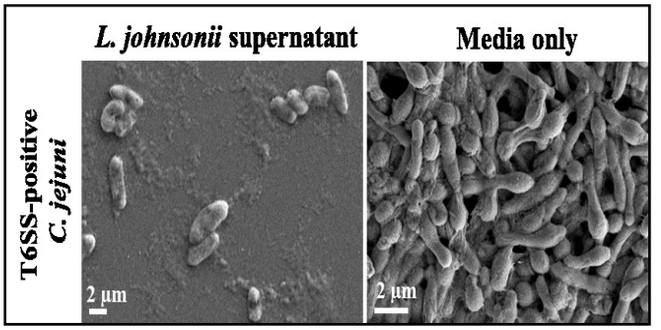


**Figure 3: *C. jejuni* biofilm adhered onto the inert surface (glass coverslips) treated with *L. johnsonii* culture supernatant. The FESEM micrographs indicate a significant reduction in biomass compared to untreated control cells/biomass of *C. jejuni* (treated with media only)**

**Co-culture with *L. johnsonii* perturbs the growth of *C. jejuni and* changes bacterial morphology:**

After confirming the functionality of the T6SS-positive strain and *L. johnsonii,* we next compared the growth profile of both bacteria when grown in an optimal growth medium. Both bacteria were grown together, and *C. jejuni* colonies were counted at different time points (5-25 h). Data presented herein suggest that the presence of *L. johnsonii* significantly reduced the growth of T6SS-positive *C. jejuni* compared to the T6SS-negative strain of *C. jejuni* (Figure 4).

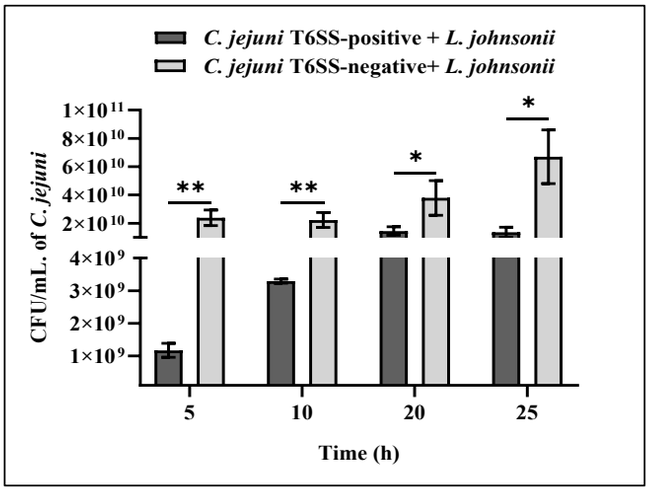


**Figure 4: Effect of *L. johnsonii* on *C. jejuni* growth. Approximately $1\times10^6$ CFU/mL of C. jejuni was used as a seeding number, while for *L. johnsonii*, $\sim1\times10^7$ CFU/mL cells were used and grown under a microaerophilic environment. The samples collected at each time point were plated onto Campylobacter selective media containing CAT selective supplement. Comparative analysis of the number of colonies (CFU/mL) that appeared on the plate suggests a significant reduction of the T6SS-positive (A) in comparison to T6SS-negative (B) strains of *C. jejuni*. *P<0.05, **P<0.01.**

To further visualize the changes specifically in T6SS-positive *C. jejuni* morphology associated with the perturbation of bacterial growth, FESEM images were captured at different post-incubation timepoints (5 h and 25 h).The FESEM micrographs suggest a significant killing of the T6SS-positive *C. jejuni* at different post-incubation times (5h and 25 h) (Figure 5).

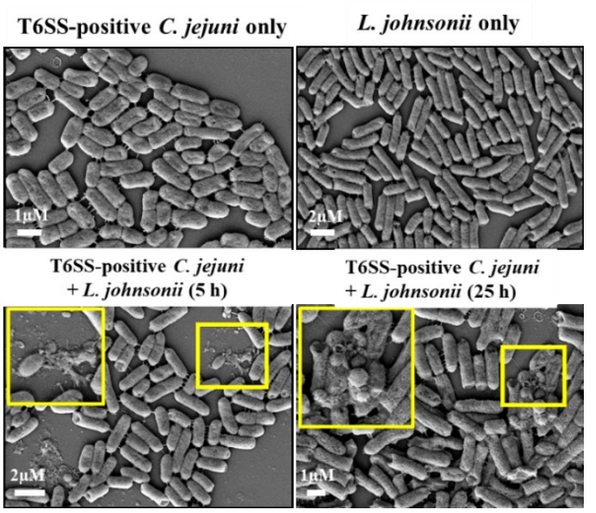


**Figure 5: FESEM micrographs of T6SS-positive *C. jejuni* co-cultured with *L. johnsonii* showing ruptured, disintegrated *C. jejuni* cells.**

***Co-culture of T6SS-positive C. jejuni with L. johnsonii to study the effect on Hcp secretion***

To check whether co-culture with *L. johnsonii* has any direct or indirect role in T6SS functionality, indirect ELISA was performed to detect the level of Hcp present in the culture supernatant. The data presented in Figure 6 indicate a slightly higher *($p<0.08$)* increase in Hcp secretion when T6SS-positive *C. jejuni* were co-cultured with *L. johnsonii*.

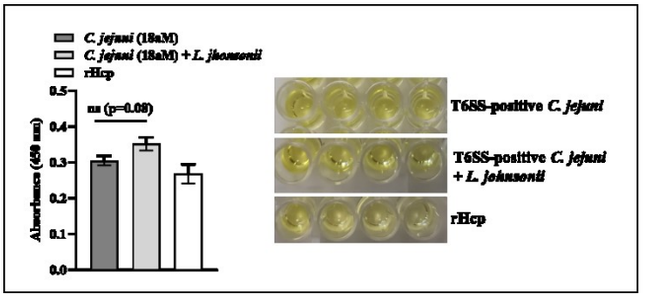


**Figure 6: Detection of effector protein (Hcp) secretion in the co-culture supernatant by indirect ELISA. TCA precipitated culture supernatant was used as a coating antigen. An anti-Hcp antibody raised in rabbits was used as primary, and HRP conjugated goat anti-rabbit IgG (H+L) served as a secondary antibody. A modest increase in Hcp secretion was recorded in the samples collected from a co-culture setup of T6SS-positive *C. jejuni* and *L. johnsonii*, compared to when *C. jejuni* was grown alone.**

The gut microbiome is a complex ecosystem where microbial interactions are critical in determining gut homeostasis (Gupta et al., 2021, 2024). Disruptions in this microbial balance can lead to dysbiosis, favouring

pathogenic bacteria over beneficial ones, which can negatively impact gut health (DeGruttola et al., 2016; Rinninella et al., 2019). Pathogenic bacteria utilize specialized secretion systems, such as the T6SS, to gain competitive advantages by injecting effector proteins into neighboring cells, aiding colonization, and evading host defences (Green and Mecsas, 2016; Pickard et al., 2017). The T6SS is found in approximately 25% of Gram-negative bacteria, including other pathogens such as *P. aeruginosa*, *V. cholerae*, and *Y. pseudotuberculosis* (Mougous et al., 2006; Wang et al., 2015; Nag et al., 2018). In *C. jejuni*, T6SS plays a key role in bacterial pathogenesis and regulating access to environmental resources (Coulthurst, 2019; Gupta et al., 2021).

While the gut harbors both beneficial and pathogenic bacteria, the T6SS in *C. jejuni* facilitates unique interactions by outcompeting other bacteria. Given that chicken is the natural host for *C. jejuni*, chicken ceca can host many *C. jejuni* harbouring fully functional T6SS as well as non-functional or T6SS-negative strains (Gupta et al., 2021). However, the mechanisms by which *C. jejuni* avoids "self-intoxication" remain unclear.

Previous studies, including ours, proposed that selective targeting of gut microbes by a functional T6SS can be perturbed by altering the gut environment (Gupta et al., 2021; 2024). Conventional therapies such as dietary supplements, faecal microbiota transplantation (FMT), and bacteriophage therapy often fail to target the gut pathogens selectively. Therefore, leveraging T6SS functionality, especially in the presence of suitable targets such as probiotic bacteria, could present a novel approach to improving gut health. This strategy could harness the natural competitive mechanisms of *C. jejuni* T6SS to selectively target pathogenic populations while keeping the beneficial microbes undisturbed in the gut ecosystem.

Towards this supposition, we chose to use *Lactobacillus johnsonii,* considering its known therapeutic benefits. Particularly *L. johnsonii* secretes a wide range of metabolites and bacteriocins such as lactic acid, hydrogen peroxide, anti-microbial peptides, and bile salt hydrolases (BSH), short-chain fatty acid (SCFA). In addition, several strains of *L. johnsonii* were found to disrupt biofilms by secreting anti-microbial peptides (bacteriocins). Hence, we first checked the potential of *L. johnsonii* in disrupting *C. jejuni* biofilm by using the culture supernatant of *L. johnsonii*. Data obtained from CV staining and confocal microscopy confirmed that biofilm disruption suggests the intrinsic activity of *L. johnsonii* metabolites or secretomes in the culture supernatant in targeting *C. jejuni* biofilm. The FESEM micrographs further revealed the disintegration of biofilm biomass and surface blebbing of *C. jejuni*.

To test the functionality of *C. jejuni* T6SS in the presence of *L. johnsonii,* we first ensured optimal growth conditions for both bacteria when co-culture. Based on the comparative analysis of the growth profile, we chose to use MH broth (without CAT selective supplement), where both bacteria can grow fastidiously.

Next, when we grow *C. jejuni* in the presence of *L. johnsonii,* with an optimal seeding number, we observed a marked reduction in the growth of the T6SS-positive strain of *C. jejuni* compared to the T6SS-negative. The enhanced killing of T6SS-positive *C. jejuni* was further visualized by FESEM micrographs of *C. jejuni* captured at different post co-culture time points (5 h and 25 h).

To look into this more critically, we checked for the amount of Hcp present in the co-culture medium. We recorded a slight increase in the Hcp level when T6SS-positive *C. jejuni* was grown in the presence of *L. johnsonii*. Although minor, the increase in Hcp production suggests the enhanced activation of T6SS in the presence of *L. johnsonii*. On the other hand, a marked reduction in T6SS-positive *C. jejuni* (compared to T6SS-negative *C. jejuni*) in the presence of *L. johnsonii* implies that functional activation of T6SS may favor an increased influx of antibacterial metabolites or other secretomes released by *L. johnsonii* via T6SS.

These findings shed some insight into how T6SS-mediated interactions can be modulated by probiotics, not only disrupting biofilms but potentially sensitizing T6SS-positive gut pathogens to environmental stressors. The observed biofilm degradation and growth inhibition indicate that *L. johnsonii* may act as a natural stress enhancer in the gut, which possibly aids in destabilizing *C. jejuni* viability. Nonetheless, the possibility that *C. jejuni* may adapt by altering its effector repertoire, attenuating T6SS activity, or acquiring resistance to anti-microbial compounds produced by probiotics remains an important consideration. Moreover, the results presented herein are performed under *in vitro* conditions using a two-species interaction model, which does not fully represent the in vivo condition, which is far more complex and involves dynamic interactions with the host immune system, mucosal surfaces, dietary influences, and the broader gut microbial community. Therefore, the translational relevance of our findings needs further studies using an appropriate *in vivo* model. Additionaly, time-course analyses, strain-specific comparisons, and functional metagenomics

could help to decipher how T6SS-mediated killing reshapes microbial networks.

Together, our study suggests that *C. jejuni*'s T6SS plays a critical role in microbial competition within the gut. However, the presence of prey or prior activation of T6SS may facilitate the influx of environmental stressors, impacting their survival. These unique attributes of T6SS, particularly its vulnerability under probiotic pressure, highlight its dual functionality: while it confers a competitive advantage, it may also sensitize pathogens to disruption. Harnessing this dynamic interaction for therapeutic use requires understanding microbial ecology and host-microbe interplay. We propose that if optimally used, these unique attributes of T6SS can be utilized as selective targeting and offer promising therapeutic approaches to improve gut health.

## Conclusion:

These findings suggest that bacterial T6SS functionality not only mediates effector-driven target killing but also induces self-depletion in the presence of specific bacterial interactors. This dual role of the T6SS may be strategically leveraged to eliminate T6SS-bearing microbial pathogens from the gut environment selectively.

## Authors' contribution:

AG performed all the experiments. SG and PB assisted in experiments and analyzed the data. AIM conceived the study and wrote the manuscript with the support of AG, SG, and PB.

## Acknowledgements:

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## Abbreviations:

T6SS: type six secretion system; FMT: fecal microbiota transplantation; PCR: polymerase chain reaction; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PVDF: polyvinylidene fluoride

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