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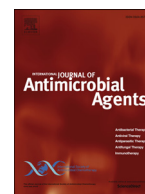
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Adaptation of *Escherichia coli* to ciprofloxacin and enrofloxacin: Differential proteomics of the SOS response and RecA-independent mechanisms

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ABSTRACT

Objective: Antibiotic resistance is a growing global healthcare challenge, treatment of bacterial infections with fluoroquinolones being no exception. These antibiotics can induce genetic instability through several mechanisms, one of the most significant being the activation of the SOS response. During exposure to sublethal concentration, this stress response increases mutation rates, accelerating resistance evolution.

Methods: To explore the role of the SOS response in fluoroquinolone adaptation, we induced *de novo* resistance by exposure to step-wise increasing concentrations *Escherichia coli* wild-type (MG1655) and a $\Delta recA$ mutant strain, which is deficient in SOS activation. Both strains were exposed to stepwise increasing concentrations of ciprofloxacin and enrofloxacin – two fluoroquinolones that differ only by a single methyl group.

Results: Development of resistance against both fluoroquinolones was severely hampered in the $\Delta recA$ mutant. While these antibiotics are often assumed to elicit similar cellular responses, our data revealed distinct genomic and adaptive differences. Building on these findings, we performed a comparative proteomics analysis to investigate how *E. coli* adapts to ciprofloxacin and enrofloxacin at the protein level.

Conclusions: The results demonstrate that the slight structural variation between ciprofloxacin and enrofloxacin leads to unique proteomic adaptations. These findings suggest that even subtle chemical differences can lead to distinct adaptive trajectories and illustrate the flexibility of cellular stress responses.

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1. Introduction

Antimicrobial resistance (AMR) remains a pressing global health crisis, with fluoroquinolones playing a central role in both human and veterinary medicine [1]. These antibiotics exert their bactericidal effects by targeting DNA gyrase and topoisomerase IV, enzymes essential for bacterial DNA replication and transcription [2]. Despite their structural similarity, individual fluoroquinolones can elicit varied cellular responses and adaptive mechanisms in bacteria, which contributes to the complexity of resistance evolution [3–5].

Enrofloxacin, widely used in veterinary practice, is metabolized into ciprofloxacin within the host [6]. This metabolic conversion adds complexity to studies of enrofloxacin's direct antibacterial effects, while ciprofloxacin, as a commonly used antibiotic in human medicine, provides a more straightforward model for studying fluoroquinolone-induced resistance [6]. Both drugs have been shown to trigger the bacterial SOS response, a critical pathway that not only drives mutation rates but also accelerates resistance development through mechanisms like horizontal gene transfer and error-prone DNA repair, mediated by the RecA protein [7–10]. This highlights the role of the SOS response as a crucial driver in the evolution of AMR.

Research into the specific adaptive responses to fluoroquinolones has largely focused on genetic changes observed through whole-genome sequencing [11]. These studies have identified key mutations in DNA gyrase, topoisomerase IV, and regulatory elements of the SOS response [12–15]. However, less

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attention has been given to proteomic changes – the shifts in protein expression and post-translational modifications (PTMs) – which could offer valuable insights into how bacterial cells rewire their metabolic and stress-response pathways under fluoroquinolone exposure [16–20]. Expanding the understanding of these proteomic adaptations may provide a more comprehensive view of the molecular underpinnings of resistance, revealing new targets for combating AMR.

Building on the observation that *Escherichia coli* exhibits distinct adaptive changes when exposed to enrofloxacin and ciprofloxacin [5], this study challenges the assumption that fluoroquinolones uniformly trigger bacterial responses. Our earlier findings highlighted the need to explore how subtle structural differences between these antibiotics influence cellular adaptations.

This research investigates the proteomic changes that occur during bacterial adaptation to enrofloxacin and ciprofloxacin, chosen for their distinct relevance in practice. Enrofloxacin is widely used in veterinary medicine, playing a pivotal role in agricultural and animal health, while ciprofloxacin is among the most commonly prescribed fluoroquinolones in human healthcare. Through evolutionary experiments coupled with proteomics analysis, this study aims to clarify the differential adaptations observed, shedding light on mechanisms driving AMR and revealing how these closely related antibiotics uniquely shape bacterial adaptation strategies.

To determine the role of the SOS response in the adaptation process, we used both wild-type *E. coli* MG1655 and a $\Delta recA$ mutant strain, which is deficient in SOS induction. These strains were subjected to stepwise increasing concentrations of enrofloxacin and ciprofloxacin, allowing us to compare their proteomic adaptations. By analysing these proteomic responses, we aim to identify specific cellular processes that underpin bacterial adaptation to these antibiotics, thus contributing to broader insights into resistance evolution and the role of the SOS response in AMR. The results emphasize the importance of considering drug-specific differences in bacterial responses. Ciprofloxacin and enrofloxacin, despite their structural similarities, induce distinct proteomic shifts. This understanding can guide the optimization of antibiotic use in diverse environments, ensuring that therapies are tailored to exploit bacterial vulnerabilities while minimizing resistance risks.

2. Material and methods

2.1. Strains, growth conditions, and antimicrobial agents

We used *E. coli* strain MG1655 as the wild-type for all experiments. The single-gene knockout mutant JW2669 ($\Delta recA636::kan$), selected from the KEIO collection, was obtained from Horizon Discovery Ltd [21]. The mutant, which harboured a kanamycin resistance cassette flanked by FLP recognition target sites, underwent cassette removal using the pCP20 method before experimentation [22].

Both wild-type and $\Delta recA$ strains were cultured in lysogeny broth containing 10 g/L NaCl, either in liquid or solid form. Initial bacterial cultures were started at an optical density of 0.1 at 600 nm (OD₆₀₀) and incubated overnight at 37°C with shaking at 200 rpm. For extended incubations over weekends, the initial OD₆₀₀ was reduced to 0.01, and the incubation temperature was lowered to 30°C to minimize overgrowth.

Fluoroquinolone stock solutions (ciprofloxacin and enrofloxacin) were obtained from Sigma Aldrich. The solutions were prepared at a concentration of 10 mM, filter sterilized, and stored at either 4°C for up to 1 week or at –20°C for a maximum of 1 month.

2.2. Minimum inhibitory concentrations (MIC)

MICs were determined twice a week for each strain, with measurements performed in duplicate using the broth microdilution method [23]. In brief, bacterial cultures were inoculated into microtiter plate wells at a starting OD₆₀₀ of 0.05. Optical density readings were taken every 10 min using a microplate reader, with 5-min shaking intervals between readings. The MIC was defined as the lowest antibiotic concentration that inhibited visible growth, indicated by an OD₆₀₀ reading of less than 0.2 after 24 h of incubation.

2.3. Evolution experiment

Evolution experiments were conducted as previously described [5,24]. Initially, MICs for both strains were determined. The starting antibiotic concentrations for each strain-antibiotic combination were set at one-quarter of the respective MICs, as detailed in the Supplementary Data (Table A1; Table A2). Following overnight incubation, the OD₆₀₀ was measured. Cultures that reached or exceeded 65% of the previous OD₆₀₀ were considered adapted, prompting a two-fold increase in antibiotic concentration in fresh medium for the next incubation. Cultures that did not meet this threshold were transferred to a fresh medium with the same antibiotic concentration as the prior incubation. Parallel cultures of each strain without antibiotics were maintained as biological controls. Three biological replicates were performed for each condition, and the experiment was completed after 30 transfers.

2.4. Proteomic analysis

Control and experimental samples included MG1655 and $\Delta recA$ strains, both untreated and treated, with three technical replicates per condition. The evolved strains were treated with the final concentration they could adapt to after 30 transfers (Table A6, Supplementary), and the susceptible strains were treated with ¼ MIC (Table A2, Supplementary). Strains were grown overnight until reaching the stationary phase. Cells were then harvested by centrifugation at 4500 × g for 10 min at 4°C. The cell pellets were re-suspended in 1 mL of lysis buffer (200 mM ammonium bicarbonate, 4% SDS, and a 5× protease inhibitor cocktail). Next, the re-suspended cultures were sonicated for 2 min and then centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was transferred to new Eppendorf tubes. An aliquot of each sample was taken for a BCA protein assay (Pierce) to determine protein concentrations. The plates were incubated at 37°C for 30 min, and the optical density was measured with a photo-spectrometer at 595 nm. Based on the assay results, the protein concentration of all samples was adjusted to 1 µg/µL, using lysis buffer, to match the sample with the lowest protein concentration. Samples were separated by reversed-phase chromatography in the Mass Spectrometry of Biomolecules Department at the University of Amsterdam using an Ultimate 3000 RSLCnano UHPLC system (Thermo Scientific, Germany) as previously described [25].

2.5. Data analysis

Data analysis and visualization was conducted in Prism 10.0.0, R, and Microsoft Excel. Overlapping proteins were analysed using UpSetR [26]. Proteomics analysis was performed using Perseus software version 1.6.14 [27]. First, quality control was performed by excluding contaminants, reverse hits, and ‘proteins identified only by site’ from the dataset, followed by log₂ transformation of protein label-free quantitation (LFQ) intensities. Experimental groups (wild-type and $\Delta recA$ mutant, exposed and not exposed to the two antibiotics, susceptible and resistant) were then established.

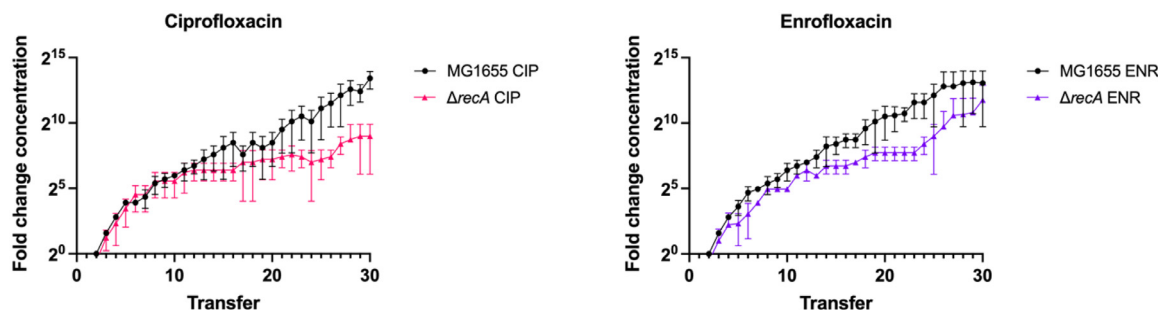


Fig. 1. Adaptation to ciprofloxacin and enrofloxacin. Comparative analysis of the adaptation to ciprofloxacin (CIP) and enrofloxacin (ENR) across multiple transfers in the two bacterial strains, MG1655 (black), and $\Delta recA$ (pink and purple) with three biological replicates for each strain. The y-axis represents the fold change in log₂ units of the antibiotic concentration, while the x-axis represents the number of transfers. Adapted from Ref. [5]. The final MIC varied between 4 and 4096 $\mu\text{g}/\text{mL}$ and can be found in Table A4 in the Supplementary.

For downstream analysis, only proteins identified and quantified with LFQ intensity values in at least half of the experimental groups were included. Annotations were added using the standard settings in Perseus. Missing LFQ intensity values were exchanged from a normal distribution (imputation) using the default settings in Perseus (width 0.3, downshift 1.8) and the most frequent values were then subtracted. ANOVA testing with permutation-based false discovery rate of 0.05 was performed to calculate the mean log₂ fold protein LFQ intensity differences between the experimental groups and adjusted *P*-values. Proteins with a *P*-value < 0.01 and a log₂ fold change below -1 or above $+1$ were considered significant.

3. Results

3.1. MG1655 and $\Delta recA$ mutant show distinct extents of adaptation

Both the MG1655 wild-type *E. coli* strain and the $\Delta recA$ knockout mutant increased tolerance to ciprofloxacin and enrofloxacin throughout 30 transfers. For ciprofloxacin, one MG1655 replicate reached a maximum concentration of 256 $\mu\text{g}/\text{mL}$, while for enrofloxacin, the wild-type strain achieved a peak concentration of 512 $\mu\text{g}/\text{mL}$ (Table A3; Table A4). In contrast, the $\Delta recA$ mutants reached maximum concentrations of 4 $\mu\text{g}/\text{mL}$ for ciprofloxacin and 32 $\mu\text{g}/\text{mL}$ for enrofloxacin.

The wild-type strain exhibited comparable adaptation rates to both antibiotics. However, the $\Delta recA$ mutants showed a slightly faster initial adaptation to ciprofloxacin, plateauing between 1 and 2 $\mu\text{g}/\text{mL}$. Up to this concentration, both strains followed a similar adaptation trajectory. Notably, two out of three $\Delta recA$ replicates exposed to enrofloxacin surpassed the 2 $\mu\text{g}/\text{mL}$ threshold, indicating variability in the adaptation potential within the knockout population (Fig. 1).

4. Susceptible not exposed

4.1. Differences in proteome between naïve wild-type and $recA$ knockout mutant

The proteomes of both the naïve knockout ($\Delta recA$) and wild-type *E. coli* strains were comparable in the absence of antibiotic exposure (Figs. 2 and 4). Principal component analysis showed that most proteins from both strains clustered together, reflecting overall similarity in relative protein abundance. In the $\Delta recA$ strain, several proteins were significantly more abundant compared to the wild-type. These included the uncharacterized proteins YdjY and YqjK, as well as BtsT (YjiY), a pyruvate/proton symporter. Other proteins that showed higher abundance in the knockout strain included LsrR, a transcriptional regulator, and BtuB, a protein involved in vitamin B12 transport. Conversely, proteins such as DacC

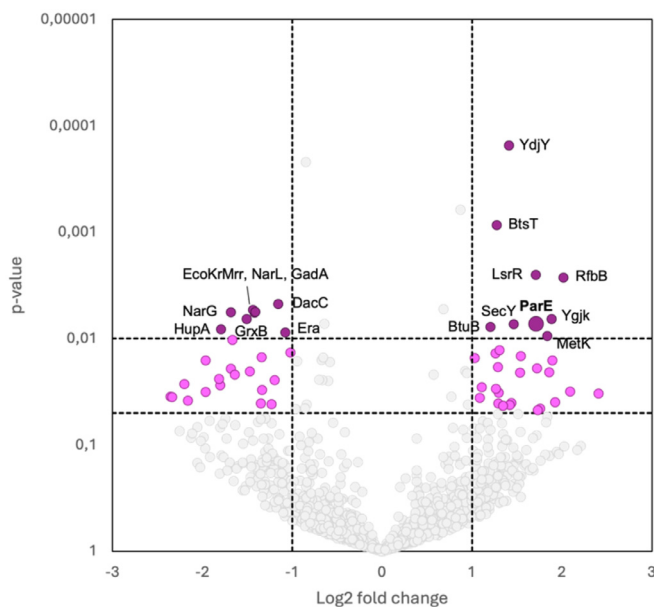


Fig. 2. Differential protein expression based on strain background – Susceptible, unexposed strains. Dark purple indicates proteins that had a log₂ fold change > 1 or < -1 and were significantly different expressed depending on the strain background ($P < 0.01$). These proteins were considered for further analysis.

(D-alanyl-D-alanine carboxypeptidase) and components of the nitrate reductase complex (NarG, NarL) were less abundant in the $\Delta recA$ strain.

Interestingly, ParE (a DNA topoisomerase and one of the targets of fluoroquinolone antibiotics) was among the proteins significantly more abundant in the knockout strain. RecA itself did not appear in the list of differentially abundant proteins, probably because it is not expressed by the wild-type strain under non-stress conditions, such as in the absence of antibiotic exposure.

The most notable separation in the dataset was driven by the expression of RecA and GadA (glutamate decarboxylase alpha, involved in acid resistance) (Figs. 1 and 3). The reduced abundance of GadA in the $\Delta recA$ mutant suggests that RecA may directly regulate the expression or stability of GadA, independent of the SOS response, revealing a novel aspect of RecA's regulatory role.

4.2. Susceptible exposed

4.2.1. Metabolic adjustments in the wild-type and $\Delta recA$ mutant to endure antibiotic stress

A comparative analysis of the proteomes from both wild-type and $\Delta recA$ knockout strains revealed significant differences in

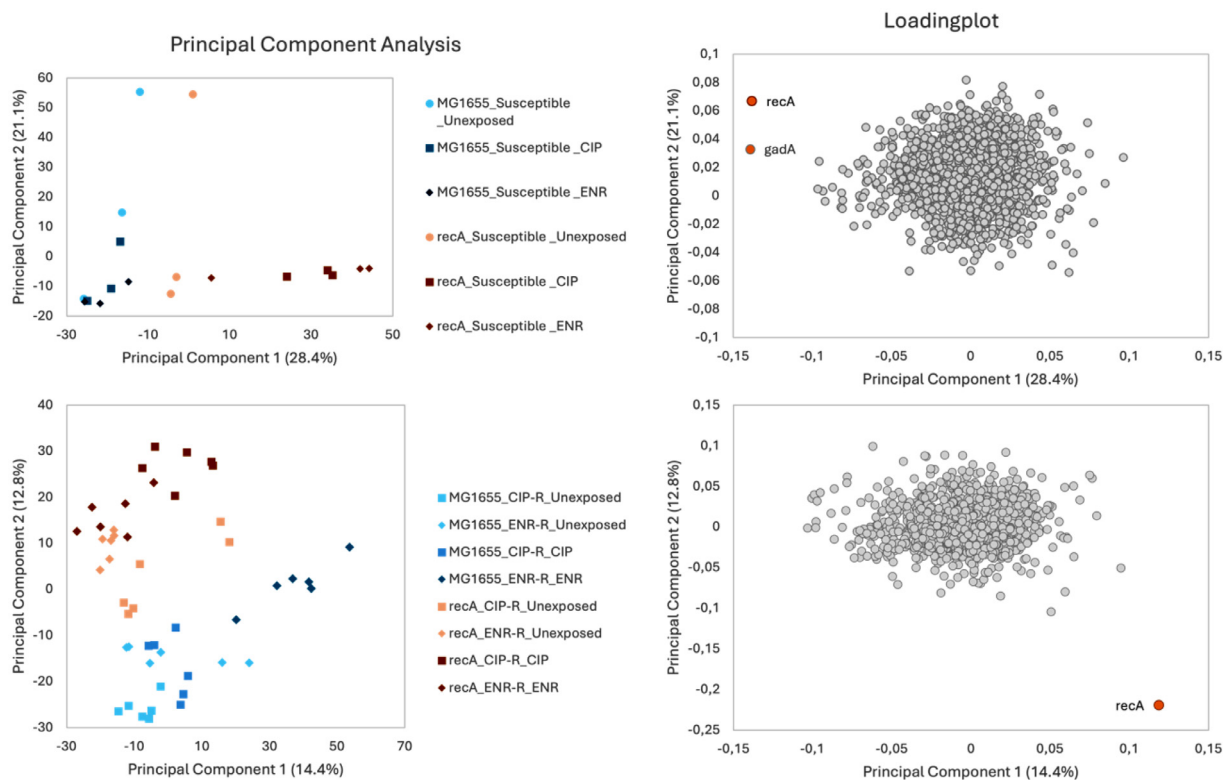


Fig. 3. Principal component analysis. The PCA plots visualize the clustering of the two bacterial strains under various conditions. The upper plots represent the susceptible (non-antibiotic adapted) samples, while the lower plots represent the adapted samples. Orange and red represent the $\Delta recA$ strains, and light blue and dark blue represent the wild-type. The biggest separation of the clusters resulted from differential RecA and GadA abundance (see plot on the top right).

pathway enrichment across the tested conditions (Fig. 4). In the susceptible wild-type strain, ciprofloxacin exposure led to a more pronounced proteomic response than enrofloxacin, involving an upregulation of glycolysis-related proteins (Figs. 4 and 5). In contrast, the knockout mutant exhibited a similar shift in proteomic activity, though glycolysis enrichment was only observed during enrofloxacin exposure. Notably, enrofloxacin induced more extensive proteomic alterations in the knockout strain compared to the wild-type.

The wild-type strain (MG1655) exhibited a broader activation of metabolic pathways in response to ciprofloxacin compared to the $\Delta recA$ mutant, highlighting the wild-type's more extensive adaptive capacity under antibiotic stress. This observation suggests that the presence of RecA, which mediates the SOS response, enables a more diversified proteomic shift, allowing the wild-type to activate a wider array of cellular mechanisms to cope with fluoroquinolone exposure. In contrast, the $\Delta recA$ mutant, lacking the SOS response, exhibited a more constrained proteomic adaptation, particularly under ciprofloxacin exposure.

No overlap in significantly differentially abundant proteins was observed between the susceptible wild-type and $\Delta recA$ strains upon antibiotic exposure (Fig. 6). This finding indicates that each strain relies on distinct proteomic adaptation strategies, likely driven by the presence or absence of RecA and the SOS response, in response to antibiotic stress.

The $\Delta recA$ mutants primarily changed abundance of proteins involved in amino acid biosynthesis, heat shock response, DNA synthesis, and metabolic pathways upon exposure to either ciprofloxacin or enrofloxacin (Table 1). This suggests that in the absence of RecA, the $\Delta recA$ strain compensates the lack of the SOS response by upregulating stress response pathways and core metabolic processes. This upregulation may be part of a mechanism to maintain cellular function under antibiotic stress without

involvement of the error-prone DNA repair processes typically associated with the SOS response.

In contrast, the wild-type strain exposed to either ciprofloxacin or enrofloxacin displayed a completely different set of significantly enriched proteins, which are associated with DNA repair, cell wall synthesis, and metabolic regulation, reflecting the wild-type's broader adaptive strategy. The presence of SOS response-related proteins like RecA, UvrA, and RarA in this list underscores the importance of DNA repair pathways and recombination in the wild-type's response to fluoroquinolones, as these processes are essential for overcoming DNA damage induced by these antibiotics.

The lack of overlap in significantly enriched proteins between the wild-type and $\Delta recA$ strains further supports the notion that each strain activates distinct cellular pathways in response to fluoroquinolone exposure. The wild-type likely leverages RecA-dependent processes, such as recombination and error-prone DNA repair, to mitigate DNA damage, while the $\Delta recA$ mutant relies more heavily on metabolic adjustments and stress response mechanisms to endure antibiotic pressure.

4.3. Adapted, not exposed

4.3.1. Antibiotic-adapted strains change their energy metabolism

Adaptation to the respective fluoroquinolones resulted in a substantial proteomic shift in both strains (Fig. 5). In the wild-type *E. coli* strain, exposure to both antibiotics led to the enrichment of proteins associated with beta-lactam resistance (Fig. 4). In contrast, the $\Delta recA$ mutant primarily exhibited a metabolic response, notably enriching oxidative phosphorylation pathways. Furthermore, adaptation to ciprofloxacin in the wild-type strain triggered a more pronounced proteomic change compared to adaptation to enrofloxacin. Conversely, the $\Delta recA$ mutant showed the opposite trend, with enrofloxacin having a greater impact on its proteome.

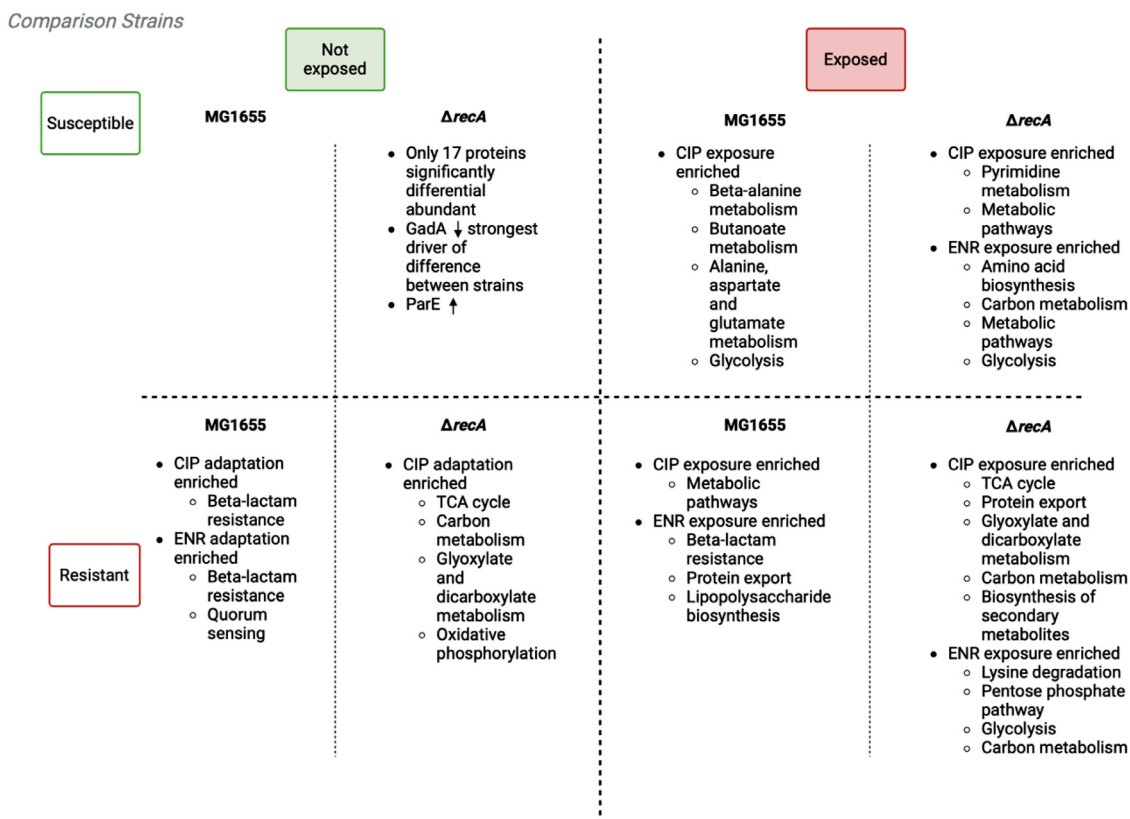


Fig. 4. Enriched pathways. Comparison MG1655 and $\Delta recA$ mutant. Analysis performed with the STRING database (<https://string-db.org>).

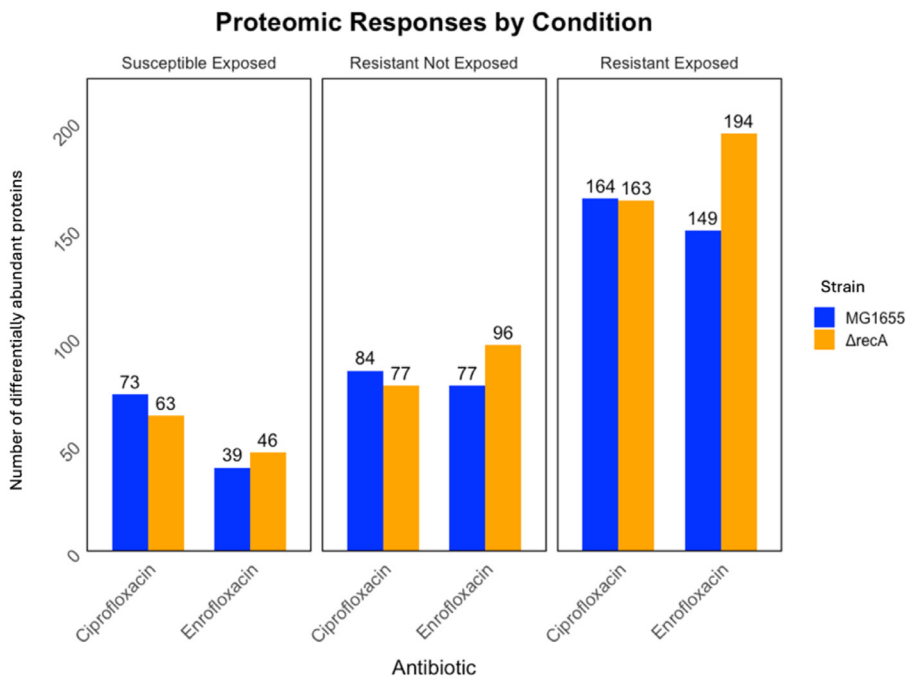


Fig. 5. Number of significantly differential abundant proteins. Comparison of MG1655 to $\Delta recA$ mutant. Blue bars represent the number of significantly more abundant proteins in the wild-type in comparison to the knockout mutant, orange bars represent significantly more abundant proteins in the knockout mutant in comparison to the wild-type.

Table 1
Significantly differential expressed proteins per intersection – Susceptible exposed.

Intersection	Gene names of corresponding proteins
$\Delta recA_CIP$ and $\Delta recA_ENR$	<i>ilvC, grpE, nrdA, asnA, sdaC, nrdB, galK, sdaB, yeeN, fklB</i>
MG1655_CIP and MG1655_ENR	<i>dacC, recA, hsdR, nirB, mdoD, ynfF, rarA, dld, uvrA, ygeV</i>

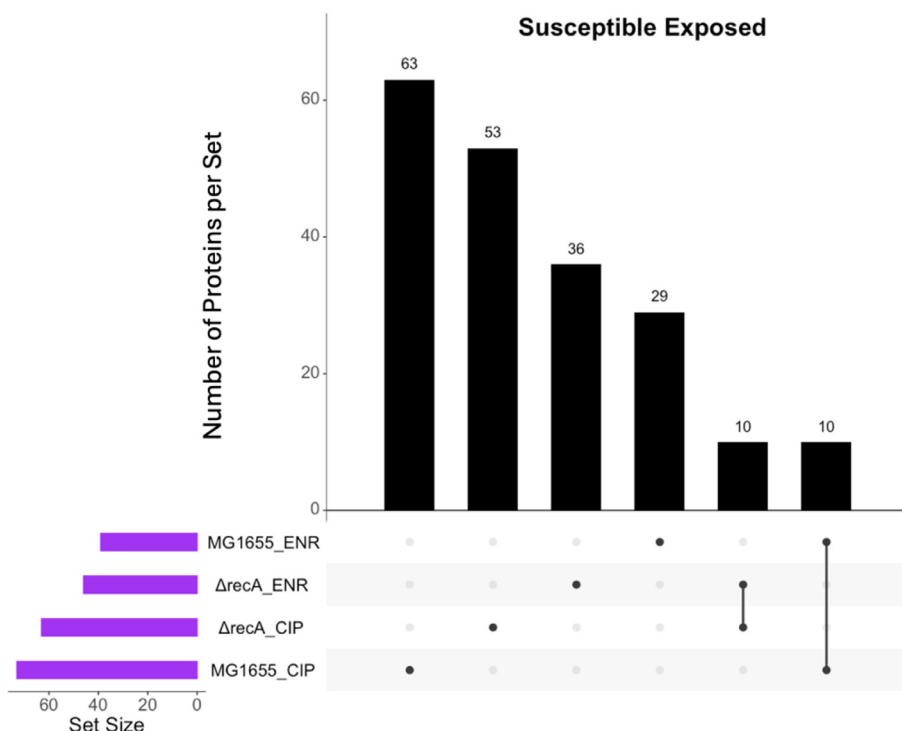


Fig. 6. Comparison of significantly differential expressed proteins per set – susceptible exposed.

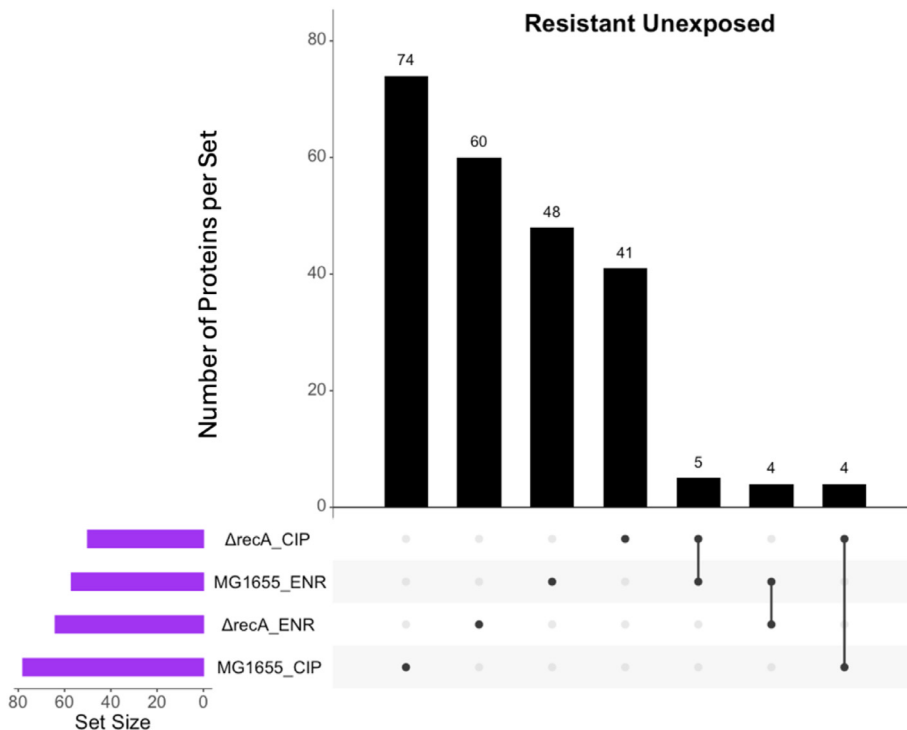


Fig. 7. Comparison of significantly differential expressed proteins per set – resistant unexposed.

While there was no overlap of significant differential abundant proteins in the susceptible exposed strains, we could observe a small set of shared proteins after fluoroquinolone adaptation (Fig. 7, Table 2). For the MG1655 strain exposed to enrofloxacin and the ΔrecA strain exposed to ciprofloxacin, the overlapping proteins are central to the tricarboxylic acid cycle and energy metabolism,

indicating that both strains may enhance their metabolic efficiency in response to antibiotic exposure. The shared proteins between the MG1655 strain and the ΔrecA mutant exposed to ciprofloxacin suggest a response to protein misfolding or stress, maintaining cell wall integrity, DNA replication, and the metabolism of nucleotides. This overlap indicates that both strains may activate pathways as-

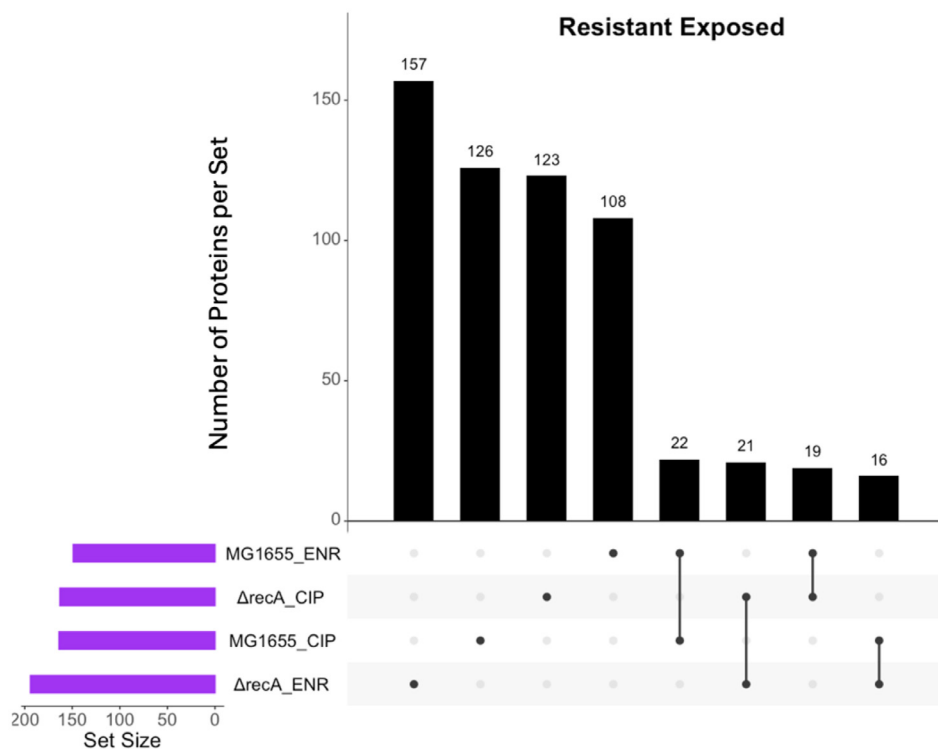


Fig. 8. Comparison of significantly differential expressed proteins per set – resistant exposed.

Table 2
Significantly differential expressed proteins per intersection – Resistant unexposed

Intersection	Gene names of corresponding proteins
MG1655_ENR and Δ recA_ENR	<i>ansB, hisJ, eco, fnt</i>
MG1655_ENR and Δ recA_CIP	<i>icd, kbl, sdhA, sucD, tktA</i>
MG1655_CIP and Δ recA_CIP	<i>groL, pal, dnaN, deoD</i>

sociated with protein quality control and cell wall maintenance during ciprofloxacin exposure, which is critical for survival under antibiotic stress.

Lastly, the overlapping proteins between the MG1655 strain and the Δ recA mutant exposed to enrofloxacin included proteins involved in amino acid metabolism and stress responses, suggesting that both strains may engage similar metabolic pathways to mitigate the effects of enrofloxacin.

Overall, the small number of shared proteins between the wild-type and Δ recA strains illustrates that while they may tap into some common pathways in response to the antibiotics, their distinct adaptive strategies likely reflect differences in their genetic makeup and regulatory mechanisms.

4.4. Adapted, exposed

4.4.1. Antibiotic-adaptation is fluoroquinolone specific

Both adapted strains exhibited similar proteomic shifts in response to ciprofloxacin exposure, whereas enrofloxacin triggered a more pronounced proteomic adjustment in the knockout mutant (Fig. 5). Remarkably, while the susceptible knockout initially showed a distinct proteomic response to ciprofloxacin, its post-adaptation response was comparable to that of the wild-type. The opposite pattern emerged with enrofloxacin exposure. Even after adaptation, the knockout mutant continued to rely heavily on proteomic adjustments as a resistance mechanism, indicating persistent stress.

The enriched pathways were similar between adapted cultures both with and without antibiotic exposure, the main difference being an increase in metabolic activity and protein export in both strains (Fig. 4). Upon enrofloxacin exposure, the wild-type strain enriched lipopolysaccharide biosynthesis proteins, potentially indicating alterations in membrane composition. Meanwhile, the knockout mutant showed increased activity in lysine degradation, the pentose phosphate pathway, and metabolic processes such as glycolysis, suggesting a metabolic shift aimed at maintaining cellular energy and resilience under stress.

Analysis of overlapping proteins per strain and antibiotic exposure revealed that the knockout mutant changed the abundance of proteins involved in oxidative stress response, nucleotide metabolism, and cellular repair processes upon exposure to either antibiotic (Fig. 8, Table 3). The wild-type, on the other hand, changed the abundance of proteins that are part of DNA repair, stress responses, and cell division. Notably, the wild-type responded to ciprofloxacin and enrofloxacin exposure by activating the SOS response, indicated by the increased abundance of RecN, DnaJ, UvrA, DnaN, RarA, MgtA, DnaG, DinD, RecX, and RecA.

5. Discussion

Despite their structural similarities, ciprofloxacin and enrofloxacin triggered distinct proteomic responses, especially in the absence of the *recA* gene. These differences in protein abundance and pathway enrichment emphasize the critical role of RecA in mediating bacterial adaptation and survival under fluoroquinolone-induced stress. Furthermore, antibiotic exposure led to an increased abundance of SOS response-related proteins in the wild-type, especially after its adaptation to the respective antibiotic. Its absence in the mutant strain profoundly altered these adaptive strategies, shifting the response from DNA repair-driven to more metabolically focused adaptation. This highlights RecA's importance in shaping cellular responses to antibiotic exposure, with

Table 3
Significantly differential expressed proteins per intersection – Resistant exposed

Intersection	Gene names of corresponding proteins
$\Delta recA$ _CIP and $\Delta recA$ _ENR	<i>sodA, nrdA, recC, hfq, ribA, rpmF, yggX, ydjA, yecD, uspA, ridA, tabA, yciO, rnf, aceK, fpr, dkgB, mdtF, ytfB, mdh, yqhD</i>
$\Delta recA$ _CIP and MG1655_ENR	<i>mrcA, fepA, lpdA, cyoA, ftsI, ppiD, acrA, secD, fflh, mreC, cirA, rfaY, rfaZ, waaU, ftsN, acrB, fdoG, yrfJ, tatB</i>
MG1655_CIP and $\Delta recA$ _ENR	<i>mtlD, ugpB, treA, rna, yciF, ydbC, ycgB, otsA, sbmC, erfK, ytfQ, yjgR, pata, yedP, rclA, yaJV</i>
MG1655_CIP and MG1655_ENR	<i>recN, fhuA, dnaJ, hsdR, uvrA, recA, udk, dnaN, rarA, mgtA, dnaG, exuR, oxyR, oppB, rmuC, polB, dinD, nfrA, recX, lasT, ybjL, yciT</i>

its absence profoundly altering the adaptive strategies employed by the mutant strain. In this respect the proteomics analysis corresponds to the genetic analysis of de novo-induced resistance to four fluoroquinolones, that showed great differences between mutations as a result of exposure to these antibiotics as well [5].

Despite the seemingly important role of RecA in fluoroquinolone resistance, the knockout strain could still survive and adapt to sub-lethal exposure, albeit less efficiently than the wild-type. This suggests alternative pathways or compensatory mechanisms that allow this strain to cope with fluoroquinolone stress. Key pathways, such as oxidative phosphorylation and other metabolic shifts observed in the $\Delta recA$ strain, could represent alternative stress responses aimed at maintaining cellular energy and redox balance. Additionally, the enrichment of proteins involved in cell wall maintenance and protein quality control indicates that the $\Delta recA$ strain may rely on general stress response systems to cope with the damage typically repaired by RecA-dependent mechanisms. These compensatory adaptations highlight the bacterial cell's flexibility in managing antibiotic-induced stress and suggest the existence of backup regulatory systems that can be activated when key players like RecA are absent. Understanding these alternative mechanisms could provide valuable insights into novel targets for inhibiting bacterial survival and resistance development, especially in strains in which traditional pathways are compromised.

The small overlap in shared differentially expressed proteins between the wild-type and $\Delta recA$ strains further suggests that, while both strains may activate some common pathways in response to fluoroquinolone stress, they predominantly rely on distinct adaptive mechanisms. Notably, despite our previous research identifying common mutations associated with fluoroquinolone resistance [5], we found no evidence that these mutations influenced the relative abundance of related proteins in either strain. This suggests that the resistance-conferring mutations do not directly affect protein expression levels. Instead, these mutations might influence protein function or stability without altering their overall abundance. This disconnection between genetic mutations and proteomic changes points to a more complex relationship between genomic alterations and resistance mechanisms, where mutations may drive structural or functional changes at the protein level without a detectable impact on their expression.

Moreover, our results revealed that the wild-type strain responded more robustly to ciprofloxacin, whereas the $\Delta recA$ mutant exhibited a stronger proteomic shift in response to enrofloxacin. This deviation from expected patterns of adaptation raises important questions regarding the specific stress responses invoked by each antibiotic and the degree to which RecA influences these pathways. These findings align with those of other studies that emphasize the role of RecA in facilitating adaptive responses to antibiotic pressure but also point to previously unrecognized RecA-independent mechanisms of survival and resistance [28–31].

Additionally, the $\Delta recA$ mutant's persistent activation of stress responses under enrofloxacin exposure, even after successful adaptation, suggests that this strain remains under substantial metabolic pressure. This ongoing stress response highlights the strain's struggle to maintain homeostasis when exposed to enrofloxacin, despite its ability to tolerate higher concentrations

compared to its ciprofloxacin-resistant counterpart. The continuous activation of stress-related pathways indicates that even the antibiotic-adapted $\Delta recA$ mutant has not fully optimized its adaptive mechanisms to manage enrofloxacin-induced stress, leaving it metabolically stressed.

The distinct proteomic responses observed between fluoroquinolones, especially in RecA-deficient strains, underscore the importance of drug-specific effects. The subtle structural differences between enrofloxacin and ciprofloxacin appear to drive differential cellular responses, even within the same organism. This variance reinforces the idea that antibiotic structure plays a pivotal role in shaping bacterial adaptation and resistance. Understanding these drug-specific responses is crucial for developing more targeted approaches to combat resistance, as it reveals that fluoroquinolones, despite their structural similarities, can impose vastly different evolutionary pressures on bacterial populations.

Furthermore, the enrichment of beta-lactam resistance-associated proteins in the wild-type strain upon adaptation to ciprofloxacin and enrofloxacin highlights an intriguing potential cross-resistance mechanism. Beta-lactam resistance proteins, such as those involved in peptidoglycan synthesis and modification, play a key role in maintaining cell wall integrity under antibiotic pressure [32]. Their upregulation in response to fluoroquinolone exposure suggests that the wild-type strain may be reinforcing its cell wall as a defensive strategy, even though fluoroquinolones primarily target DNA replication.

This proteomic modification shows that, in addition to responding directly to DNA damage, the wild-type strain might activate broader stress responses, including mechanisms typically associated with resistance to other classes of antibiotics. Such cross-protection could provide a survival advantage, as the strain not only addresses the immediate threat posed by fluoroquinolones but also enhances its defence against potential disruptions in cell wall synthesis. The activation of beta-lactam resistance proteins may represent a broader adaptive strategy where bacteria leverage overlapping resistance pathways to enhance survival under varying antibiotic pressures. This finding aligns with the growing understanding that antibiotic resistance is often a multifaceted process, where bacteria can employ overlapping and interconnected resistance mechanisms to counteract diverse forms of antimicrobial stress.

A key finding in this study involves GadA, an enzyme critical to *E. coli*'s acid-resistance system, which has been previously linked to antibiotic resistance [33]. Strains resistant to amoxicillin, enrofloxacin, and tetracycline all upregulate *gadA*, indicating its central role in bacterial adaptation to antibiotic stress [34]. The transcriptional activator GadE is pivotal in this process, particularly under enrofloxacin exposure, and the absence of GadE severely impairs the strain's ability to adapt, underscoring its importance in resistance mechanisms.

Our findings further verify and extend this connection. In the $\Delta recA$ mutant, the impaired SOS response reduced GadA abundance, which may partially explain the strain's reduced ability to adapt to fluoroquinolone exposure. This compromised response, in combination with the absence of RecA, likely weakens the mutant's overall ability to mount a robust defence against fluoroquinolone stress.

These results underscore the critical interactions between RecA, GadE, and GadA in regulating the stress response and facilitating fluoroquinolone resistance. The dysfunction of these key regulatory proteins in the $\Delta recA$ mutant might contribute to its reduced capacity to develop resistance, contrasting with the more robust adaptive mechanisms observed in the wild-type strain.

While this study provides valuable insights into the proteomic adaptations of *E. coli* strains exposed to fluoroquinolones, additional adaptations may be considered. First, this study focused on changes in protein abundance without investigating PTMs. PTMs, such as phosphorylation or acetylation, can significantly influence protein function and bacterial stress responses. A more comprehensive analysis incorporating PTMs could provide a fuller understanding of how fluoroquinolone exposure affects bacterial adaptation.

Furthermore, the experiments were conducted in controlled laboratory conditions, which may not fully mimic the complexities of natural environments, such as biofilm formation, varying oxygen levels, or interactions with other microbial species. The in vitro setting limits the direct application of these findings to clinical or environmental scenarios.

Lastly, the study focuses on a single wild-type *E. coli* strain (MG1655) and a corresponding *recA* knockout mutant. Given the genetic diversity of *E. coli* strains, as well as the diversity of bacterial species that are exposed to fluoroquinolones, the findings may not be universally applicable. For example, a previous study investigating proteomic changes in ciprofloxacin-exposed clinical isolates revealed significant genomic and proteomic variability among the strains [35]. Such variability underscores the importance of expanding this research to include other *E. coli* strains, as well as different bacterial species.

6. Conclusion

This study underscores the importance of RecA in mediating robust proteomic adaptations to fluoroquinolone exposure, particularly in response to enrofloxacin. The wild-type strain displayed a broader range of adaptive mechanisms, including pathways associated with cell envelope reinforcement and multidrug resistance, while the *recA* knockout mutant relied heavily on core metabolic pathways to survive. The differential response to ciprofloxacin and enrofloxacin, despite the structural similarity of these two antibiotics, reveals an unanticipated complexity in how *E. coli* adapts to fluoroquinolone stress, suggesting that slight differences in drug structure can significantly alter bacterial adaptation strategies. These findings highlight the need for a deeper understanding of the molecular mechanisms behind antibiotic resistance and could inform the future development of more effective antibiotic therapies.

Sequence information

Sequences have been deposited.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT (OpenAI, 2023) to improve readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Data availability

All mass spectrometry data are available in the MassIVE repository (<https://massive.ucsd.edu/>) via the dataset identifier **to be announced**.

announced. All other original data can be obtained from the corresponding author.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ijantimicag.2024.107420](https://doi.org/10.1016/j.ijantimicag.2024.107420).

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