FINAL REPORT
Project ID: K 140349

BIOLOGICAL AND GENETIC BASIS OF EPIDEMIOLOGICAL COMPETITIONS BETWEEN *SALMONELLA* ENTERITIDIS AND *S.* INFANTIS

The primary objective of this project was to investigate the epidemiological and genetic mechanisms driving the predominant dissemination of *Salmonella* Infantis, in Hungarian broiler flocks gradually replacing *S.* Enteritidis since the early 2000s.
In this report, the results are presented in the order of the three Work Packages of the project, each based on the central hypothesis that the diversity of multidrug-resistant (MDR) mobile genetic elements (primarily plasmids) and the potential interaction of *Salmonella* with *E. coli* play a key role in the above changes observed in *Salmonella* serovar composition.

WP1. Diversity of characteristic resistance/virulence plasmids of *S*. Enteritidis, *S*. Infantis and commensal *E. coli* and their epidemiological relevance.

First part of our working hypothesis was that *S.* Enteritidis, *S.* Infantis and commensal *E. coli* represent the three key players in the molecular epidemiologic events leading to the emergence and predominant spread of MDR *S.* Infantis in broilers in Hungary and several other European countries. The aim of WP1 was to compare genomic diversity of plasmid borne resistance/virulence determinants of *S.* Enteritidis, *S.* Infantis and *E. coli*, and to reveal genomic and functional diversity of specific resistance/virulence plasmids with a potential for intraspecies or intergeneric circulation in broilers.

1.1. Comparative MDR collection of S. Infantis and cohabitant strains of commensal E. coli from broilers.

To establish the basic collection of strains, 263 caecal and 209 faecal samples were processed for the isolation of broiler strains of *Salmonella* and *E. coli*. These caecal and faecal samples represented 162 and 162 broiler flocks respectively, and were included in the national surveillance system for *Salmonella*. *S.* Infantis was detected in 8-21% of the broiler samples, of which the caecum was the most contaminated source of isolation. None of the samples tested were positive for *S.* Enteritidis.
Salmonella-positive caecal samples served as a common source for the simultaneous isolation of *S.* Infantis and *E. coli* strains, which were designated here as cohabitant strains. Each sample was used to isolate multiple strains of *S.* Infantis and cohabitant *E. coli*, to represent the diversity of antimicrobial resistance (AMR) phenotypes identified for different sample sources. The AMR phenotype was determined against a set of antibiotic compounds selected to detect plasmid-mediated phenotypes.
Resistance phenotyping resulted in the selection of 24 strains of *S.* Infantis strains and 97 cohabitant *E. coli* strains, representing the diversity of AMR/MDR phenotypes in broilers. Overall, *S.* Infantis strains were characterized by a low resistance diversity, with the predominant MDR phenotype of nalidixic acid-sulfonamide-tetracycline (Nal-Sul-Tet), indicating the predominance of the large plasmid of *S.* Infantis pSI54/04 (Szmolka et al., 2018). The resistance diversity of *E. coli* was approximately three-fold higher than that of S. Infantis, and strains with ampicillin-ciprofloxacin-tetracycline (Amp-Cip-Tet) phenotypes were most frequently identified. Only a few strains were identified as pansensitive to both the species (Szalai N., MSc thesis, 2022).
Because poultry represent the major sources of human *Salmonella* infection, a representative set of human strains of *S.* Enteritidis (n=91) and *S.* Infantis (n=78) were included as a cohort, representing clinical samples from the National Public Health Surveillance System. The Nal-Sul-Tet resistance phenotype was also characteristic of human *S.* Infantis strains, whereas only

two strains of *S.* Enteritidis were resistant to ampicillin. The *E. coli* collection was completed with commensal and pathogenic (ExPEC) MDR strains isolated earlier from the faeces and bone marrow of broiler chickens and day-old chicks.

1.2. Core genome diversity of Salmonella and E. coli strains from broilers.

To meet the overall goals of WP1, molecular characterization of the above representative strains of the two *Salmonella* serovars and cohabitant *E. coli* was performed including detection and typing of mobile resistance genes and class 1 integrons. Based on the results of the genotyping, whole genome analysis of selected 14 S. Infantis and 57 cohabitant *E. coli* strains representing 14 caecal samples, and 24 human strains of *S.* Enteritidis was performed. For this, we first compared the discriminative power of different WGS-based bioinformatics platform, in the comparative analysis of pre-emerging (pansensitive) and emerging (MDR) *S.* Infantis genomes of Hungarian and international distribution.
The results indicated the most conservative nature of core genome analysis in contrast to cloud gene analysis, which proved to be the most sensitive genome analysis. This method was able to clearly differentiate *S.* Infantis clones and lineages in Hungary from those of other geographical regions, as well as differentiate *S.* Infantis strains representing different epidemiologic periods in Hungary (Nagy/Szmolka et al., 2020). These findings represent important baseline data for genome analysis of contemporary genomes of *Salmonella* and *E. coli*. The genomic diversity of *E. coli* and S. Infantis strains was characterized by core genome (cg) MLST, with special focus to the epidemic PFGE clone B2 of *S.* Infantis, which is epidemic in Hungary. cgMLST analysis showed a high genomic heterogeneity of *E. coli* and provided the first insight into the genomic epidemiology of the MDR clone B2 of *S.* Infantis with the plasmid pSI54/04 (Szmolka et al., 2021).

1.3. Mobile genomes of S. Enteritidis, S. Infantis and cohabitant strains of E. coli.

Exploring the genomic diversity of *S.* Infantis, *S.* Enteritidis and *E. coli* was primarily based on the comparative characterization of the mobile resistomes and virulomes, using web-based tools for *in silico* detection of acquired antibiotic resistance genes and plasmid replicon types.
Mobile resistome analysis of the core collection of cohabitant of *E. coli* showed a large diversity of acquired resistance genes and plasmid types, with the highest prevalence of $bla_{\text{TEM-1}}$ and *tet*(A) for ampicillin and tetracycline resistance. Plasmid-encoded genes *qnrS* and $bla_{\text{CMY-2}}$ conferring resistance to high-priority antibiotics such as fluoroquinolones and ESBL-s were relatively frequent in broiler strains of *E. coli*. Co-existence of multiple plasmid types was detected therefore, no correlation between certain resistance genes and plasmid types could be established (Szmolka et al., 2021).
In contrast to *E. coli*, most broiler and human strains of *S.* Infantis and *S.* Enteritidis carry a reduced set of resistance genes and were identified as monoplasmidic. Frequently detected genes such as *aadA1*, *sul1* and *tet*(A) are known to be associated with IncI plasmid pSI54/04 of *S.* Infantis, indicating the continuous circulation of this plasmid in broiler populations. For S. Enteritidis, the pSEV virulence plasmid of IncF type was mostly identified. The ampicillin resistance $bla_{\text{TEM-1}}$ gene was associated to IncX plasmids in both serovars.
Overall, this is the first comparative genomic analysis of contemporary broiler strains of S. Infantis and *E. coli* in Hungary. The analysis of cohabitant strains of *E. coli* and *S.* Infantis allowed to gain first insight into the potential interplay of resistance genes between these two enteric bacteria. Comparative analysis of the mobile resistomes pointed to only a few plasmid types and mobile resistance genes that could be considered as potentially transferable between E. coli and S. Infantis. Among these, *tet*(A), $bla_{\text{TEM-1}}$ and *qnrS1* genes and IncI1 plasmids could

have the greatest contribution to the microevolution and genetic interaction between these two species (Szmolka et al., 2021).

WP2. Contribution of resistance plasmids to the persistence, environmental survival and epidemiological competition between serovars.

These studies were conducted to reveal possible biofilm-associated differences between serovars and estimate the significance of specific resistance and virulence plasmids in biofilm formation, assuming that increased biofilm formation could lead to increased persistence and more effective dissemination of *S.* Infantis in broiler environment.

2.1. Biofilm formation in Salmonella and E. coli: A comparative analysis.

For this, the biofilm analysis of a selected set of 114 strains of *S.* Enteritidis, *S.* Infantis and cohabitant *E. coli* with representative AMR genotypes was performed at three different temperatures: 20°C, 28ºC and 37ºC. The biofilm morphotypes were determined on Congo Red (CR) agar plates, after incubation for 96 hours, whereas the quantitative biofilm assay was conducted in 96-well polystyrene plates under identical incubation conditions (Genevaux et al., 1996). Finally, a specific collection of *S.* Infantis and cohabitant *E. coli* strains was established to reveal their interactions in dual-species biofilms. Subsequently, several S. Infantis-E. coli strain combinations were formed between the cohabitant strains, and tested in parallel with the single-species biofilm at the same incubation conditions.
The results indicated that there were no significant differences in biofilm morphotypes between the species/serovars studied. Predominantly, the rdar (red, dry, and rough) and pdar (pink, dry, and rough) colony morphologies were detected with similar prevalence for both *Salmonella* and *E. coli* strains. For several strains, the colony morphotype changed from rough to smooth with increasing temperature, a change particularly notable in *S.* Infantis.
This complex multi-species comparison study demonstrated that the increasing temperature negatively influenced biofilm formation of both *E. coli* and *S.* Enteritis on polystyrene surfaces. *E. coli* biofilm activity varied the most with temperature, whereas MDR strains of S. Infants consistently demonstrated high biofilm production at all temperatures tested. Overall, the biofilm-forming capacity of MDR *S.* Infantis was approximately threefold higher than that of *S.* Enteritidis, and twofold higher than that of commensal *E. coli* from broilers (Fodor Zs., MSc thesis, 2023). These findings support our hypothesis that the enhanced environmental survival due to robust biofilm activity may contribute to the spread and dominance of this serovar over *S.* Enteritidis in broiler populations.
Considering the biofilm activity of the strains in dual-species cultures, the competitive behavior of *S.* Infantis and *E. coli* resulted in a significant (>50%) reduction of overall biofilm production in the majority of the strain combinations. This reduction in biofilm production of dual-species biofilms compared to that of individual strains, can be explained by various genetic and environmental factors (such as quorum-sensing, metabolic-, or receptor competition) that may interfere with optimal biofilm formation for both species, but now provides intriguing questions for further studies presently outside our scope.

2.2. Relation between the biofilm production and antimicrobial resistance.

To reveal the complexity of the mechanisms underlying biofilm production in MDR strains of *E. coli* and *Salmonella*, it is necessary to assess whether biofilm formation capacity correlates with increased antimicrobial resistance, or even with the presence of specific AMR plasmids. According to the intensity of biofilm production at 28°C, the strains were grouped into three categories: strong, medium and weak. The MICs (minimal inhibitory concentration) for

ampicillin (Amp), cefotaxime (Ctx), ciprofloxacin (Cip) and tetracycline (Tet) were determined, selection based on the characteristic mobile AMR/MDR phenotypes of the tested strains. Assembled contig sequences were mapped to a set of 19 reference genes (including *adr, csg, flic/fljB, glg* and *pga*) involved in biofilm forming pathway described for *E. coli* (https://www.genome.jp/entry/map02026).
Results indicated, that 46% and 43% of the *E. coli* and *Salmonella* strains respectively, were regarded as strong biofilm producers, while 63% of the *S.* Enteritidis strains were assigned to the weak producers. Overall, a high level of resistance to ampicillin and tetracycline was detected, with MIC_{90} values of 256 and 128 respectively, for both species, but we found that the elevated MIC did not correlate with strong biofilm formation. On the other hand, we identified the biofilm polysaccharide synthesis operon *pgaABCD,* to be associated with *E. coli* strains with strong biofilm activity. Furthermore, of the tested biofilm marker genes, the FliC/FljB flagellin gene *fljB* was only detected in *S.* Infantis strains, and its presence was not specifically related to increased biofilm formation.

2.3. Testing biofilm-associated functions of characteristic antimicrobial resistance plasmids, with special regard to pSI54/04.

Our further working hypothesis was that, certain hybrid plasmids (with resistance and virulence genes) such as the pESI-like *tet*(A) plasmid pSI54/04 of *S.* Infantis (Szmolka et al., 2018) could contribute to the persistence and environmental survival of the strains. Although, the WGS-based comparison revealed a relatively narrow interface between the mobile resistomes of E. coli and of *S.* Infantis, the resistance genes *tet*(A), *bla*$_{TEM-1}$ and *qnrS1* and their corresponding plasmids conferring resistance to tetracycline, ampicillin and ciprofloxacin were representative of both species (Szmolka et al., 2021). To test the contribution of these plasmids to biofilm formation, specific collections of *S.* Infantis (n=2), *E. coli* (n=6) and *S.* Enteritidis (n=2) strains with the above plasmids were subjected to plasmid elimination.
Traditional plasmid curing resulted in the production of strains lacking the *tet*(A), *bla*$_{TEM-1}$ and *qnrS1* genes in two of the six *E. coli* strains. However, the elimination of the *tet*(A) plasmid pSI54/04 and the *bla*$_{TEM-1}$ plasmids of *S.* Infantis and *S.* Enteritidis failed in spite of using combinations of incubation temperatures and treatment with different concentrations of acridine orange, ethidium bromide or SDS (Paul et al., 2020). Therefore, a CRISPR/Cas12 system, targeting the tetracycline resistance *tet*(A) gene and the origin of replication *ori*V was specifically designed to eliminate plasmid pSI54/04 of *S.* Infantis. The CRISPR/Cas12 technique allowed the production of a CRISPR-Cas12 transformant strain of S. Infantis with a truncated pSI54/04 plasmid, lacking resistance and transfer regions of ~74 kb. in size.
To test the contribution of the MDR plasmid pSI54/04 to biofilm production, multiple comparisons were performed between i) the plasmidic/non-plasmid wild-type strains SI54/04 and SI69/94 of *S.* Infantis (Szmolka et al., 2018), ii) SI54/04 and pSI54/04 transconjugant strains of S. Infantis and *E. coli*, and iii) SI54/04 plasmidic and the promising CRISPR-Cas12 transformant strain (with the truncated plasmid). The results revealed a fourfold difference between the biofilm production of the pSI54/04-plasmidic strain and that of strain SI69/94 without the plasmid. The transfer of pSI54/04 resulted in an approximately two-fold increase in the biofilm activity of the transconjugant strains of *S.* Infantis and *E. coli*, whereas the deletion of the MDR region had no significant effect on biofilm activity. These results indicate that the interplay between the chromosome and specific AMR/MDR plasmids contributes to the complex regulation of biofilm activity.

WP3. Intraspecies and intergeneric plasmid exchanges potentiating E. coli as a reservoir for plasmids

The aim of these studies was to describe the characteristic AMR plasmids of *S.* Infantis and Enteritidis and to estimate their significance in the competition between the two serovars. According to our working hypothesis, that commensal *E. coli* strains could be regarded as reservoirs for resistance plasmid towards to *S.* Infantis, we further aimed to test contribution of cohabitant *E. coli* as potential donors to the emergence of characteristic plasmids of *S.* Infantis.

3.1. Complete plasmids of Salmonella and E. coli with potential epidemic significance

Genome analysis of broiler strains of *S.* Infantis revealed the prevalence of IncI1 plasmids associated with the tetracycline resistance gene *tet*(A) and the virulence genes *fyuA* and *irp* (tellurium resistance/Yersiniabactin siderophore) in 80% of the strains. This hybrid (resistance/virulence) genotype, indicated the uninterrupted predominance of the MDR plasmid pSI54/04 in recent populations of *S.* Infantis. In a few strains the coexistence of pSI54/04-like plasmids with the *bla*$_{\text{TEM-1}}$-*qnrS1* plasmid of IncX1 type was identified. S. Enteritidis strains lacked determinants for mobile AMR, except for two strains, that were resistant to ampicillin and also carried *bla*$_{\text{TEM-1}}$ gene on an IncX1 plasmid. These AMR and hybrid plasmids were regarded as representative of our comparative *Salmonella* collection from broilers and humans. Reconstruction of complete plasmid genomes was performed using nanopore sequencing.
The plasmid sequence of pSI54/04 (278 kb.) was annotated based on the complete sequence of Israeli human megaplasmid pESI (Cohen et al., 2020) which was used as a reference. Genomic comparison revealed, that the Hungarian and the Israeli epidemic plasmids of *S.* Infantis showed a sequence identity of 99.8%, the difference consisted of an approximately 18 kb region carrying a class 2 integron in the structure of pESI.
AMR plasmids of IncX1 type are widespread among enteric bacteria and have a versatile genomic architecture. IncX1 plasmids of *S.* Infantis and *S.* Enteritidis (50 kb and 38 kb respectively) shared low (<75%) sequence identity. The *bla*$_{\text{TEM-1}}$ gene was commonly identified in IncX1 plasmids of both serovars, but *qnrS1* gene was only detected in *S.* Infantis. Here we confirmed the epidemiological importance of IncX plasmids, by providing the first evidence of the emergence of a high-risk resistance mechanism and characterizing the complete mcr-1 colistin-resistance plasmid in a poultry *E. coli* strain in Hungary (Szmolka et al., 2023).
By characterizing complete plasmids representing the AMR diversity of *S.* Infantis, *S.* Enteritidis and *E. coli*, we contribute to establishing the first Hungarian complete plasmid collection available as a reference for further comparative genomic studies on these serovars and species.

3.2. Interaction between serovars and species: competition and plasmid exchange.

In relation to the interaction between the *Salmonella* serovars Infantis and Enteritidis, two plasmids are considered to be of epidemic relevance: the tetracycline/MDR resistance *tet*(A) plasmid pSI54/04 of *S.* Infantis, and the ampicillin resistance *bla*$_{\text{TEM-1}}$ plasmid of *S.* Enteritidis. *In vitro* growth competition experiments were performed to reveal the contribution of these AMR plasmids to the growth and competitive potential of the strains. In the competition assay, each serovar was represented by a resistant (Amp or Tet) and a sensitive (S) strains. Mixed cultures were assembled as follows: i) *S.* Infantis^{S} + *S.* Enteritidis^{Amp}, ii) *S.* Enteritidis^{S} + *S.* Infantis^{Tet}, iii) *S.* Infantis^{Tet} + *S.* Enteritidis^{Amp}. The growth potential of individual strains was also tested in single cultures.

The results showed that strains of *S*. Infantis outgrew *S*. Enteritidis in all experimental settings, independent of plasmid content. The MDR strains of S. Infantis showed significantly ($p<0.05$) higher growth potential than those without the plasmid pSI54/04. Furthermore, we found that, plasmidic strains of *S*. Infantis were able to outcompete *S*. Enteritidis in mixed cultures, and showed significantly better growth in mixed cultures than in single cultures. These observations, together with those related to the increased biofilm activity of *S*. Infantis compared to *S*. Enteritidis, further support our suggestion that these traits could have a decisive contribution to the emergence of MDR *S*. Infantis strains and to their competitive success against *S*. Enteritidis in Hungarian broiler flocks.

We further aimed to determine the transfer potential of these characteristic plasmids between the serovars and laboratory strains of *E. coli*. The plasmid transfer was performed by conjugation on both solid and liquid media using a D:R ratio of 1:1 and coincubation at 37°C. Transconjugants were selected using rifampicin and plasmid transfer was confirmed by PCR and plasmid profile analysis.

Results shown that none of the plasmids were transferable to *E. coli* under the above conditions. Similarly, none of the recipients of either serovar were able to take up these plasmids. Interestingly however, the plasmid $bla_{\text{TEM-1}}$ was transferable from *S*. Enteritidis to the MDR *S*. Infantis carrying the plasmid pSI54/04, but not vice versa. This suggests increased susceptibility of *S*. Infantis to the uptake of plasmids from *S*. Enteritidis, which could further improve the epidemiological success of this serovar.

In relation to the interaction between *E. coli* and *S*. Infantis, comparative resistome analysis of the cohabitant strains revealed only a few mobile resistance genes that could be considered potentially transferable between *E. coli* and *S*. Infantis. Based on this, we selected pairs of cohabitant *Salmonella* and *E. coli* strains with these genes in common and performed in vitro conjugations under the conditions described above. For the interaction between the two species, *Salmonella* strains were used as recipient to test the role of *E. coli* as a potential plasmid donor for *Salmonella*.

The results showed that while plasmids *tet*(A), $bla_{\text{TEM-1}}$, and *qnrS1* were conjugative from *E. coli* to the laboratory *E. coli* strain, none were transferable from *E. coli* to *S*. Infantis. Thus, this finding slightly rewrote our initial hypothesis on the potential *E. coli* origin of plasmid pSI54/04 of *S*. Infantis, and the role of *E. coli* in the emergence of *S* Infantis as a MDR serovar in broiler flocks.

Summary

The primary objective of this project was to investigate the epidemiological and genetic mechanisms driving the predominant dissemination of multidrug-resistant (MDR) *Salmonella* Infantis in Hungarian broiler flocks, which have been gradually replacing *S*. Enteritidis since the early 2000s. The study was structured into three Work Packages (WPs) to explore the hypothesis that the diversity of MDR mobile genetic elements, particularly plasmids, and the interaction between *Salmonella* and *E. coli* are crucial factors in the serovar change.

WP1 focused on characterizing resistance and virulence plasmids in *S*. Enteritidis, *S*. Infantis, and commensal *E. coli*. It was found that S. Infantis exhibited a low diversity of resistance phenotypes, predominantly carrying the MDR plasmid pSI54/04. In contrast, *E. coli* demonstrated a higher resistance diversity. Whole-genome analysis revealed significant genomic heterogeneity and specific resistance genes such as *tet*(A) and $bla_{\text{TEM-1}}$ were common in both *E. coli* and *S*. Infantis.

WP2 investigated the role of biofilm formation in persistence and dissemination. *S.* Infantis showed significantly higher biofilm production than *S.* Enteritidis and *E. coli*, which may contribute to its increased environmental survival and prevalence.

WP3 investigated plasmid exchanges between species and serovars. While characteristic plasmids were not transferable between *E. coli* and *S.* Infantis under experimental conditions, *S.* Infantis demonstrated a higher competitive and growth potential than S. Enteritidis, independent of plasmid content.

Conclusions

The study successfully identified key genetic and epidemiological factors contributing to the predominance of MDR *S.* Infantis in Hungarian broiler flocks. The presence of the pSI54/04 plasmid, along with enhanced biofilm formation, were significant contributors to the persistence and competitive success of *S.* Infantis over *S.* Enteritidis. Although *E. coli* was shown to have a high diversity of resistance genes, its role as a direct plasmid donor to S. Infantis was limited under the study conditions. These findings provide critical insights into the mechanisms driving the emergence of MDR *S.* Infantis and offer a foundation for developing strategies to mitigate its spread in poultry populations. Further research is warranted to explore the complex interactions and potential for resistance gene transfer between these bacterial species in different environmental conditions.

Expected benefits of the reported results for innovation as well as for broiler production and international trade of Hungarian broiler meat.

One of the findings of this project is the discovery of a high biofilm forming capacity of *S.* Infantis stains, which was essentially higher than that of *S.* Enteritidis. This trait is definitely an advantage of the *S.* Infantis for a higher tenacity (environmental survival) and is giving the objective of the Industry, to produce and elaborate more effective cleaning and disinfectant toool and technologies for the broiler industry against *S.* Infantis.
Our results on the WGS based comparative genome analysis of Hungarian and international strains of *S.* Infantis from different geographical regions provided excellent counter argument and clarification against claims of some EU countries against imported Hungarian broiler meat saying that *S.* Infantis strains might have contaminated their human population. In the paper of Nagy/Szmolka et al., (2020) we proved these claims are not supported by the results of our comparative core-, and cloud gene analysis. This paper is now acknowledged and highly cited.
The observation about inhibition of biofilm formation of S. Infantis in the dual (*Salmonella*/*E. coli*) system might be a starting point for further research to work out competitive colonization systems against *S.* Infantis by commensal *E. coli* (similarly to the use of *E. coli* Nissle 1917 strain for humans).

Closing notes/remarks

The original timetable of this project has been changed three times. First the project was extended by one year due to the Covid-19 crisis, moving the deadline to November 2023. During this period, the Institute (VMRI) underwent significant financial and administrative reorganization as it regained partial independence within the ELKH in April 2022. Consequently, a new finance group had to be formed, causing substantial challenges in maintaining routine purchasing and accounting procedures. This led to especially long delays in public procurements, as detailed in our extension requests dated October 24, 2023, and January 2024.

These documents also report that the administrative and financial difficulties prompted a young PhD student and a newly trained technician to leave the VMRI for better pay and higher job security. This departure left the project leader without technical support for several months and under pressure to recruit suitable personnel.

All these administrative, financial, and personnel issues have significantly hindered the research work on this project. The project leader is grateful for the granted extensions, which have been immensely helpful, though they could not fully resolve all the mentioned difficulties and problems.

Despite these challenges, it can be concluded that the main goals of the project have been achieved, as reported.

Publications and MSc theses with the support of this project

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