MOLECULAR GENETIC SELECTION TO REDUCE SALMONELLA TYPHIMURIUM CIRCULATIONS IN CHICKENS

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Summary
Salmonella infection in chickens continues to be a major public health concern. Among *Salmonella enterica* serovars*,* Typhimurium is the cause of severe intestinal pathology in young chicks and economic losses for the poultry industry. Additionally, *S.* Typhimurium could infect humans and result in an acute gastrointestinal infection. Controlling *S. enterica* in poultry industry is an ongoing concern for several countries around the world. Increasing the genetic resistance of chicken to salmonella by genetic selection programs, which may be carried out using phenotypic or genotypic data, is an efficient way to prevent salmonellosis. The first generations of simple crossing between Fayoumi (F) and Rhode Island Red (R) breeds and their ½F½R and reciprocal ½R½F crosses were utilized to estimate heterotic effects. Also, the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to detect polymorphic associations of gallinacin *(Gal)* genes *(Gal2, Gal3, Gal4* and *Gal5)* with caecal bacterial count of *S.* Typhimurium*,* and IgA, IgG and IgM antibody titres in the studied populations. The chicks of each genetic group (120 birds) were infected with *S.* Typhimurium at 10 day of age (10^6 cfu/chick). The R breed had a significantly higher least square mean of *S.* Typhimurium load, followed by ½F½R cross, ½R½F cross, and F breed while, The F breed had the highest LSMs of IgA, IgG, and IgM antibody titres, followed by ½R½F cross, ½F½R cross, and the R breed. Direct heterosis estimates were significant for *S.* Typhimurium count and IgA antibody titre, but not for IgG and IgM antibody titres. For molecular associations, the CC genotype of *Gal3* gene had a significant lower *S.* Typhimurium count and higher IgA and IgM antibody titres than TT genotype in R breed, while the birds of the genotype TC had lower significant *S.* Typhimurium count in ½R½F crossbred and significant higher antibody titres in ½F½R crossbred. In the *Gal4* gene, birds with the GG genotype had a lower significant *S.* Typhimurium count and IgA, IgG and IgM antibody titres than birds with the AA genotype in R breed, but birds of the genotype AG had higher significant IgA, IgG and IgM antibody titers in ½R½F crossbred than the birds of GG and AA genotypes. Birds with the CC genotype of *Gal5* gene had lower significant *S.* Typhimurium count and higher IgA and IgG antibody titers. The birds with genotype AA had lower significant *S.* Typhimurium count and higher significant IgA and IgG antibody titers than birds with CA genotype. This study suggested that the Fayoumi breed may be employed in breeding programs to boost immunity against *S.* Typhimurium in chickens, and the *Gal3*, *Gal4*, and *Gal5* genes might be used as marker-assisted selection in chicken selection programs to increase *S.* Typhimurium resistance.

INTRODUCTION

Salmonella enterica is among the most common bacteria infecting chickens. It is a Gram-negative, facultative anaerobe microbe that is flagellated and rod-shaped. *S. enterica* infection in chickens can occur at any age, but hatched chicks are more sensitive to the infection (Gast, 1997). *Salmonella enterica*, *Salmonella bongori*, and *Salmonella subterranea* are the three species included within the genus *Salmonella*. Human salmonellosis is mostly due to the consumption of contaminated chicken meat and eggs. As a result, the occurrence of salmonella infections in chicken flocks and their management are still major public health concerns (Yang et al., 2019). *S. enterica* (*S.*) serovars Enteritidis and Typhimurium can survive in the chickens' gastrointestinal tract (GIT), particularly in the caecum. In birds, *S. enterica* produces both systemic and asymptomatic infections (Li et al., 2018).

Vaccination is a frequently used method of disease prevention. Producers that have a little number of poultry rarely vaccinate them. These circumstances put small-scale chicken farms at risk of infection, which could lead to the contamination of meat and eggs. This problem could be overcome by selecting chicks that are resistant to the infectious pathogens. Enhancing chicken health is a fundamental objective of poultry breeding, and crossbreeding is one of the strategies for leveraging genetic variety. Genetic polymorphism is becoming more relevant as a source of genetic markers in several sectors of chicken breeding. Characterization of molecular markers, as well as functional genomic approaches, could be used to improve immune responses against Salmonella in chickens. Researches on resistance to *Salmonella* spp. in poultry have showed that the genetic effects are significant (Kramer et al., 2003; Iraqi et al., 2011; Zhang et al., 2020). Molecular technologies are being used as a new frontier for identifying molecular markers to be employed in marker-assisted selection procedures in order to achieve larger genetic gains in crossbreeding programs (Wakchaure et al., 2015).

Gallinacin genes 1 to 13 were localized on chromosome 3 in chickens, and each gallinacin gene has the same genomic structure of four short exons separated by three different length introns (Xiao et al., 2004). Gallinacin transcription has been found to be abundant in cells involved in the innate immune system response to microbial pathogens (Ganz, 2003; Xiao et al., 2004), and they have antibacterial efficacy against both Gram-positive and Gram-negative bacteria (Higgs et al., 2005). The proposed activity mechanism consists of the initial binding to the bacterial membrane through electrostatic interactions, followed by the penetration within the cytoplasmatic environment and, finally, the interaction with the machinaries devoted to the RNA, DNA and protein synthesis, blocking them (Hasenstein and Lamont, 2007; Zhang et al., 2020).

The haplotypes of the immune-related genes have shown strong correlations with the immune response against salmonella in chickens (Hasenstein et al., 2006; Legarra et al., 2011; Ardiyana *et al.*, 2020). However, there have been few studies about the relationship between gallinacin genes and immunological characteristics in chickens. In an effort to go deeper into some of these ideas, the main objectives of the current study to estimate direct heterosis on caecal *S.* Typhimurium bacterial count and antibody titers of IgA, IgG, and IgM in crossing

between Fayoumi and Rhode Island Red chickens, to determine associations of *Gal2, Gal3, Gal4* and *Gal5* genes with *S*. Typhimurium bacterial count, and IgA, IgG, and IgM antibody titers.

MATERIAL AND METHODS
Crossbreeding
A crossbreeding experiment was conducted between Fayoumi (F) and Rhode Island Red (R) to obtain ½F½R cross and its reciprocal ½R½F cross. Chicks of F, R, ½F½R and ½R½F populations (120 birds from each genetic group) were inoculated with *S*. Typhimurium at 10 days of age (10^6 colony-forming units (cfu) in 1 ml per chick).

Examination of the bacterial count in the caecum and antibody titres
At the 10th week of age, 24 birds from each group's caecum were sampled for bacteria using *S*. Typhimurium culture and quantification methods (Saleh et al. 2021). In order to detect the antibody titres, blood samples were collected in the 4th week of age from 12 chicks from each population. Antibody titres were detected using the Calbiotech Inc. (CBI) Salmonella spp. IgA, IgG, and IgM ELISA Kits (Cat#: ST093G 96 Tests).

Blood sampling, DNA extraction and PCR-RFLP
For the PCR-RFLP assays, 96 blood samples from the four genetic groups of chickens were obtained (24 samples from each population of F, R, ½F½R and ½R½F chicks). The primers, DNA extraction process, polymorphic assessment of genetic immune response of *Gal2, Gal3, Gal4* and *Gal5* genes, and PCR-RFLP test for genotyping SNPs of Gal genes on chromosome 3 utilizing *Hpy*-CH4IV, *Ava*I, *Alu*I, and *Hinf*I restriction enzymes, along with the procedures, have been previously described (Saleh et al., 2021).

Statistical models for estimating heterotic effects and polymorphic associations
Data of *S*. Typhimurium bacterial count and IgA, IgG, and IgM antibody titers were analyzed using the animal model and the estimation of heterotic effects as described by Saleh et al. (2022). Also, the animal model used to detect the polymorphic associations among genotypes of *Gal* genes and immunity traits was described by Saleh et al. (2021).

RESULTS
Populations means
The generalized least square means (GLMs) for *S*. Typhimurium count bacterial count and antibody titers were presented in Figure 1. The results showed that the bacterial counts of *S*. Typhimurium in R breed were highly significant, followed by ½F½R cross, ½R½F cross and F breed, while the means of IgA, IgG, and IgM antibody titers in F breed were highly significant, followed in descending order by ½R½F cross, ½F½R cross, and R breed.

Figure 1. Generalized least-square means for *Salmonella* Typhimurium count and antibody titers as affected by genetic group of chicks. R= Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R= Fayoumi × Rhode Island Red; Different letters indicate significant differences at p<0.05; cfu= colony forming units; OD= optical density.
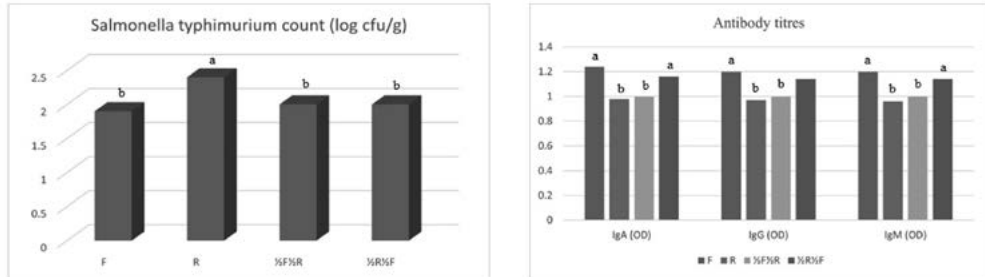
Heterosis effects
The heterotic effects were significant for *S.* Typhimurium count, and for IgA antibody titer, but non-significant for IgG and IgM antibody titers (Figure 2). In addition, the heterotic percentages for *S.* Typhimurium count and IgA antibody titer were significant (p< 0.01).

Figure 2. The generalized least square solutions of heterotic effects for microbiological and immunological traits in crossing Fayoumi with Rhode Island Red chickens. Percentages of heterosis computed as {Estimate of in heterosis units/[(F+R)/2] x100}; ns = non-significant; ** = significant differences at p<0.01; cfu= colony forming units; OD= optical density.
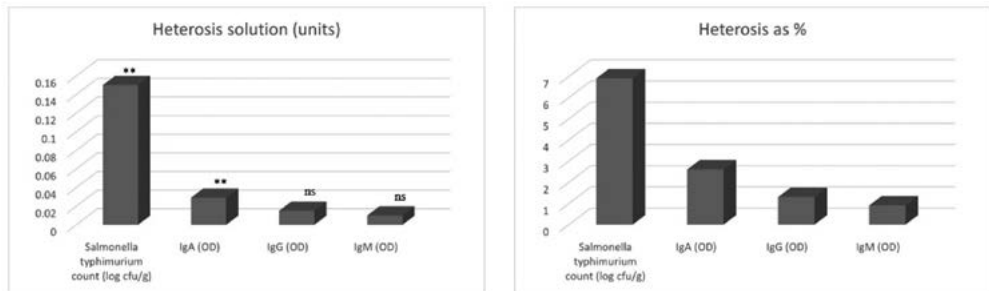

PCR amplification and digestion
PCR products of 583, 664, 600 and 623 bp in size for *Gal2, Gal3, Gal4* and *Gal5* genes respectively, were amplified in the four studied populations. The PCR-RFLP digestion of PCR products in parents and F_1 progeny using the *Hpy*Ch4IV restriction enzyme for *Gal2* gene were monomorphic and included two fragments of 388 and 195 bp (CC genotype). For the *Gal3* gene, the PCR-RFLP digestion with *Ava*I produced fragments including a single uncut fragment of 664 bp (TT genotype; the common homozygote), two fragments of 443 and 221 bp (CC gen-

otype; the minor homozygote) and three fragments of 664, 443 and 221 bp (TC genotype; the heterozygote). For the *Gal4* gene, the *AluI* restriction enzyme produced fragment sizes of 600, 416 and 184 bp, the major homozygote was AA and the AG genotype was the heterozygote, while the GG genotype was the minor homozygote. For the *Gal5* gene, the PCR-RFLP products of 623, 402 and 133 bp digested by the *Hinf*I restriction enzyme represented the major CC homozygote, the CA heterozygote and minor AA homozygote.
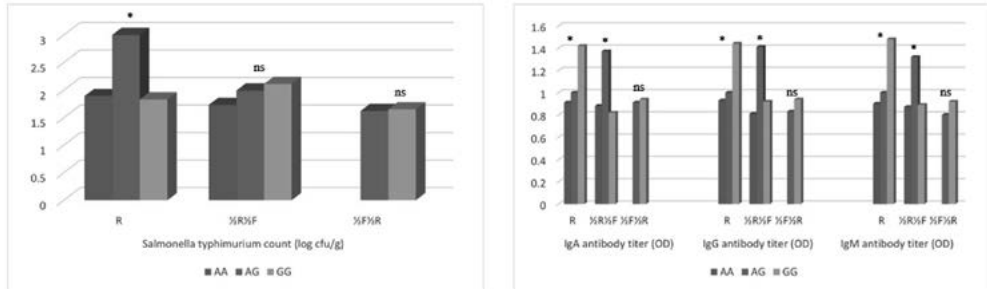
Polymorphic associations between the genotypes of Gal genes and studied traits
The *Gal2* gene in all populations was monomorphic and *Gal3, Gal4* and *Gal5* genes were monomorphic in the F breed, so they were excluded from the association analysis. The genotypes of *Gal3* gene were associated significantly with *S*. Typhimurium count, along with IgA, IgG and IgM antibody titres (Figure 3). The genotype CC of *Gal3* gene breed had lower significant *S*. Typhimurium count and higher IgA and IgM antibody titers than TT genotype in R and ½R½F chickens. The TC genotype in ½R½F crossbred had a lower significant *S*. Typhimurium count than TT genotype and there were non-significant differences between the TC and CC genotypes for *S*. Typhimurium count between, while the TC genotype had higher IgA and IgM antibody titres than CC in ½F½R crossbred.

Figure 3. Generalized least square means for immune traits as affected by SNP genotypes of the *Gal3* gene in all studied populations. R= Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R= Fayoumi × Rhode Island Red; ns = non-significant; * = significant differences at p<0.05; cfu= colony forming units; OD= optical density.
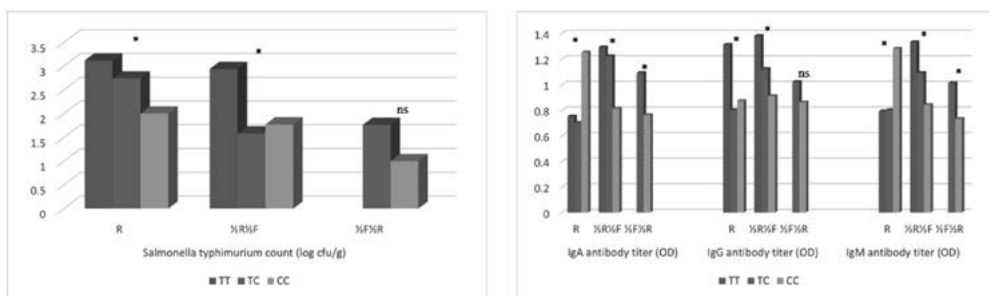

The SNP genotypes of *Gal4* gene were associated significantly with *S*. Typhimurium count and IgA, IgG and IgM antibody titres (Figure 4). The genotype GG had lower *S*. Typhimurium count and higher IgA, IgG and IgM antibody titres than AA genotype in R breed. There were not significant differences between the genotypes of *Gal4* for *S*. Typhimurium count in ½R½F crossbred, while, the genotype AG had higher significant IgA, IgG and IgM antibody titres than AA and GG genotypes in ½R½F crossbred. There were not significant differences between the AG and GG genotypes for *S*. Typhimurium count and IgA, IgG and IgM antibody titres in ½F½R crossbred.

Figure 4. Generalized least square for immune traits as affected by SNPs genotypes of *Gal4* gene in all studied populations. R= Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R= Fayoumi × Rhode Island Red; ns = non-significant; * = significant differences at p<0.05; cfu = colony forming unit; OD = optical density.

There were significant associations between the genotypes of *Gal5* gene and *S.* Typhimurium count, and IgA, IgG, and IgM antibody titres (Figure 5). The genotype CC had significant lower *S.* Typhimurium count, and higher IgA and IgG antibody titres in R breed. Significant differences between the genotypes of *Gal5* were not observed for *S.* Typhimurium count, and IgA, IgG, and IgM antibody titres in ½R½F crossbred. The birds with AA genotype had lower significant *S.* Typhimurium count, and higher IgA and IgG antibody titres in ½F½R crossbred.

Figure 5. Generalized least square means for immune traits as affected by SNPs genotypes of *Gal5* gene in all studied populations. R= Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R= Fayoumi × Rhode Island Red; ns = non-significant; * = significant differences at p<0.05; cfu = colony forming unit; OD = optical density.
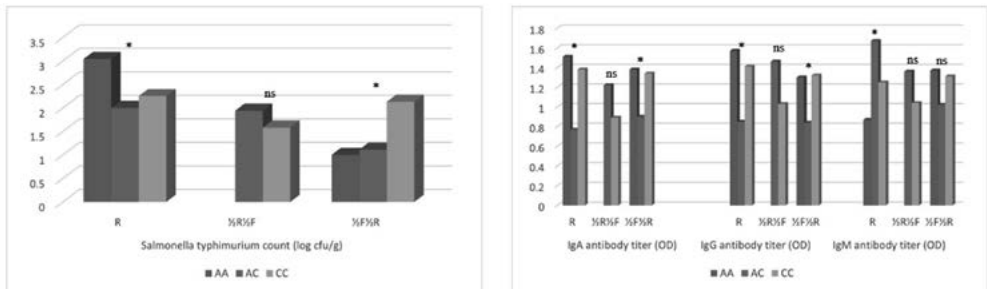
DISCUSSION

The generalized least square means indicated that the F breed had lower *S.* Typhimurium count and higher antibody titres. These findings revealed that the F breed was more resistant to Salmonella than the R breed. Gebrerufael et al. (2015) reported that there was a difference in the susceptible to salmonellosis between the local and F breeds, and the Koekoek breed had a significantly higher *S.* Typhimurium count than the local and F breeds. In comparison to Leghorn and other breeds, the F are regarded less vulnerable to several poultry infections such as *Salmonella* spp., *Eimeria*

spp., Marek's disease airus and avian influenzavirus (Cheeseman, 2007; Schilling et al., 2019). All those suggests that crossing F and R breeds could improve the immunity responses in broilers against *S.* Typhimurium. The estimates of heterosis previously reported by Iraqi et al. (2011) indicated significant reduction in *Salmonella* cells count associated with significant increase in antibody titers at 4th week of age. The current research specifically investigated the polymorphic associations of four *Gal* genes to caecal *S.* Typhimurium count and antibody titers in F, R, ½F½R and ½R½F chickens. Those genes are functionally equivalents to the mammalian beta-defensins and are crucial for chickens' innate defenses against bacterial pathogens (Hasenstein et al., 2006). There were significant differences between the genotypes of *Gal3, Gal4* and *Gal5* gene for *S.* Typhimurium count, and IgA, IgG, and IgM antibody titres in R, ½F½R and ½R½F chickens. Hasenstein and Lamont (2007) demonstrated that *Gal1, Gal2, Gal4, Gal7, Gal8, Gal9* and *Gal10* had no significant relationships with caecal bacterial load in chicks from an intercross line. Zhang et al. (2020) found that the five SNPs detected in *Gal5* were strongly associated with sensitivity to *Salmonella spp*. The association among haplotypes and IgA, IgG and IgM antibody titers against *S.* Typhimurium may provide useful information to select chicks with superior adaptive immune response. According to the reported findings, the genotypes of the *Gal3, Gal4* and *Gal5* genes could be utilized for marker-assisted selection in breeding strategies, one of which was to increase hens' resistance to *S.* Typhimurium.

CONCLUSION
Crossing F and R resulted in a favorable heterotic reduction in *S.* Typhimurium bacteria load. The ½R½F cross might be utilized to boost chick resistance against *S.* Typhimurium, which would be accompanied by higher antibody titers and lower *S.* Typhimurium colonization. The molecular associations indicated that utilizing the SNP markers (T222C, A188G, and C80A in the *Gal3*, *Gal4*, and *Gal5* genes, respectively) may aid in the identification of genotypes with the best potential for improving chicken immune traits against *S.* Typhimurium and growth performance.

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