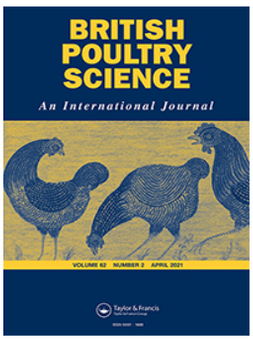

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Polymorphic characterisation of gallinacin candidate genes and their molecular associations with growth and immunity traits in chickens

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ABSTRACT

1. Four gallinacin (*GAL*) genes were assessed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) to characterise these genes in Fayoumi (F) and Rhode Island Red (R) breeds and their crosses of Rhode Island Red × Fayoumi (½R½F) and Fayoumi × Rhode Island Red (½F½R).
2. Genes examined were *GAL2, GAL3, GAL4* and *GAL5*. The molecular associations between the SNPs of the gallinacin genes and body weight, caecal bacterial count and the serum antibody titres of IgA, IgG and IgM were determined. In the R breed, the frequency of TC genotype was higher than TT and CC genotypes for the *GAL3* gene. The GG genotype frequency was higher than AA and AG genotypes for the *GAL4* gene in the other genetic groups, and the CA genotype frequency was higher than CC and AA genotypes in crosses for the *GAL5* gene.
3. In all populations, the frequency of the C allele was higher than the T allele for the *GAL3* gene, the G allele was higher than the A allele for the *GAL4* gene and the C allele was higher than the A allele for the *GAL5* gene. The observed heterozygosity in R, ½R½F and ½F½R was 0.476, 0.375 and 0.158 for the *GAL3* gene, 0.458, 0.615 and 0.250 for the *GAL4* gene and 0.053, 0.792 and 0.739 for the *GAL5* gene, while the expected heterozygosities were 0.490, 0.430 and 0.145 for the *GAL3* gene, 0.430, 0.348 and 0.219 for the *GAL4* gene and 0.229, 0.478 and 0.496 for the *GAL5* gene, respectively.
4. On a molecular level, the genotype TT was significantly higher for body weight than TC and CC genotypes in the *GAL3* gene. Birds with the GG genotype had a significantly lower *Salmonella typhimurium* count than birds with AA genotype in the *GAL4* gene. Birds with the genotype AA had higher significant body weights than those with CC and CA genotypes in the *GAL5* gene.
5. The results indicated that the *GAL3, GAL4* and *GAL5* genes are potential candidates for selection programmes to improve *S. typhimurium* resistance and body weight in chickens.

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Chickens; gallinacin genes; PCR-RFLP associations; body weights; immune traits

Introduction

Genetic polymorphism plays an increasingly important role in providing genetic markers in many sectors of poultry breeding (Saxena and Kolluri 2018). Molecular markers, based upon DNA sequence, could be used for breeding high-performance meat and eggs lines in the poultry industry (Kulibaba and Podstreshnyi 2012). Molecular marker characterisation along with functional genomic methods provide opportunities for enhancing genetic improvement programs in chickens (Gao et al. 2007). Single nucleotide polymorphisms (SNP) have been used in association studies, but PCR-RFLP, as a rapid, low cost technique, has been used to assess SNP in candidate genes responsible for a variety of physiological functions in chickens (Hasenstein et al. 2006; Hasenstein and Lamont 2007; Khatab et al. 2017; Liu et al. 2018).

In the last two decades, several studies have reported associations between immune genes and growth, immune response, bacterial burden and antibody titres against *Salmonella spp*. in chickens (Kramer et al. 2003; Khatab et al. 2017; Saleh 2019; Thinh et al. 2019). Research on potential candidate genes and their use in selection programmes for improving immunity, bacterial load and antibody titres response and for increasing genetic resistance against *Salmonella spp*. has been published (Tohidi et al. 2013; Muhsinin et al. 2017; Ardiyana et al. 2020; Zhang et al. 2020). In chickens, *GAL1-13* genes have been mapped within an 86-kb region of chromosome 3 and these are abundant in cells that are involved in the innate immune system response against microbial infections (Xiao et al. 2004). These gallinacin genes could be used to exhibit a wider range of antimicrobial activity against both gram-positive and gram-negative bacteria (Higgs et al. 2005). The first study on *GAL* genes was performed by Hasenstein and Lamont (2007) who identified and analysed *GAL1-13* genes for associations with the response to *Salmonella spp*. count in chickens.

For the above mentioned concepts, the main objectives of the present study were: (1) to characterise the polymorphism in gallinacins 2, 3, 4, and 5 candidate genes of Fayoumi (F) and Rhode Island Red (R) chickens and their crosses (½R½F and ½F½R) using the PCR-RFLP technique, and (2) to detect associations between immune gallinacin candidate genes and body weight, caecal *S. typhimurium* count, *E. faecium* bacterial counts and serum antibody titres.

Materials and methods

All experimental procedures involving animals handling and treatment were approved by Faculty of Agriculture at

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Supplemental data for this article can be accessed here.

Moshtohor, Benha University, Egypt (Approval Number: 2016–1).

Experimental birds

F and R breeds and their crosses (½R½F and ½F½R) were used to assessed the polymorphic association of gallinacin genes (*GAL2, GAL3, GAL4* and *GAL5*). The details of the crossbreeding experiment between F and R breeds and management of the studied genetic groups used have been described by Saleh et al. (2020).

Caecal bacterial samples and S. typhimurium and E. faecium examination

The bacterial strain of *S. typhimurium* was obtained from the Animal Health Research Institute, Agriculture Research Centre, Giza, Egypt. The *E. faecium* was supplied by the Food Safety and Biotechnology Laboratory, Regional Centre for Food and Feed, Agriculture Research Centre, Giza, Egypt. The bacterial counts analyses were carried out in the laboratory of the Research Park at the Faculty of Agriculture, Benha University. Salmonella and Shigella (SS) and De Man, Rogosa and Sharpe (MRS) agar were used in the identification and isolation of bacterial strains. Chicks from each genetic group (120 birds) were divided into three groups (40 bird per group), with 40 chicks from the first group allocated as the control group, while the treated group were inoculated with *S. typhimurium* and challenged with *E. faecium* at seven and 10 days of age (10^6 colony forming units (cfu) with 1 ml oral inoculation per chick). The bacterial samples were collected from the caeca of 24 birds from each group at 10 weeks of age, according to the procedure described by Kaiser and Lamont (2001). Fifteen chicks from each genetic group were randomly chosen and examined bacteriologically to ensure the absence of *Salmonella spp*. Cloacal samples were collected using sterile cotton swabs that were moistened in phosphate buffered saline, whereby the tip was carefully inserted and rotated in the cloaca of the chicks. The swab was kept in a sterile tube containing 10 ml of buffered peptone water and transported to the laboratory approximately 2 h after collection, stored under refrigeration, and processed on the day of sampling. The pre-enrichment of the samples in non-selective medium was done at a dilution of 1:10 in buffered peptone water. This dilution was incubated for 24 hours at 37°C, and 100 µl of the pre-enriched culture was plated on SS agar. The plates were incubated for 24 hours at 37°C, and then examined to ensure the absence of *Salmonella spp*.

An aliquot of 1 g caecal material was serially diluted in 9 ml of sterile saline solution and 1 ml of bacterial suspension was pipetted into a dilution tube containing 9 ml of phosphate buffered saline; making a 10^1 dilution. Tenfold serial dilutions were made to obtain 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 per ml, and 1 ml from each dilution was plated on SS and MRS agar (Titan biotech ltd.). The plates were incubated for 24 hours at 37°C, and colony forming units (cfu) were counted. The viable cell counts were expressed as cfu/surface area, calculated using the following formula (Zelver et al. 1999; Herigstad et al. 2001):

Log (average cfu/drop vol.)(dilution factor)(Vol. scrapped into/surface area)

The lowest numbers of *S. typhimurium* and *E. faecium* recovered from the plate count procedure was 100 cfu (Kaiser and Lamont 2001). At 10 weeks of age, 24 chicks from each genetic group were slaughtered by cervical dislocation. Caecal content suspension was measured by using a thermo Orion pH metre after calibration at pH 4.0, 7.0 and 10.0.

Measuring serum antibody titres

Blood samples were collected from 12 chicks in each genetic group at four weeks of age for measuring the antibody titres using ELISA tests. The Calbiotech Inc. (CBI) IgA, IgG and IgM ELISA Kits Cat#: ST093G (96 Tests) were used for detecting IgA, IgG and IgM antibody titres against *S. typhimurium*.

Blood sampling, DNA extraction and PCR-RFLP

Ninety-six blood samples were collected from the four genetic groups of chickens (24 samples from each genetic group of F, R, ½F½R and ½R½F birds) and were used in PCR-RFLP analyses. For blood sampling, primers, DNA extraction and polymorphic assessment of genetic immune response of *GAL2*, *GAL3*, *GAL4* and *GAL5* genes, PCR-RFLP assay for genotyping SNPs of *GAL* genes on chromosome 3 using *HpyCH4IV*, *AvaI*, *AluI* and *HinfI* restriction enzymes were described by Saleh et al. (2020).

Characterisation of different genetic groups

Allelic and genotypic frequencies were calculated and the SNP $T>C_{196}$, $T>C_{222}$, $A>G_{188}$ and $C>A_{80}$ located in the intronic region of *GAL2, GAL3, GAL* and *GAL5* genes, respectively, were assessed by calculating the effective number of alleles (Ne), the observed (Ho) and the expected (He) heterozygosity using GENALEX software, version 6.5 (Peakall and Smouse 2012). The polymorphism information content (PIC) was calculated using CERVUS software, version 3 (Kalinowski et al. 2007). The F-statistics of pairwise genetic differentiation among the populations (F_{ST}), heterozygosity due to inbreeding for each locus (F_{IT}) and the reduction in heterozygosity due to inbreeding within each population (F_{IS}) were calculated using GENEPOP software, version 3.4 (Raymond 1995, http://genepop.curtin.edu.au/).

Model of polymorphic associations

The following animal model was used to detect the polymorphic associations among genotypes of *GAL* genes and body weights or immunity traits using the PEST software (Groeneveld 2006):

$$y = Xb + Z_a u_a + e$$

Where y = the vector of observations of body weights and immunity traits; b = the vector of fixed effects of genetic groups (four levels), bacterial treatments (three levels), sex (males and females), and *GAL* gene genotypes (three genotypes for each SNP separately); (X) and (Za) = incidence matrices corresponding to fixed and additive random effects of the birds (u_a), respectively; e = the residual error. The solutions of b were calculated by the method of generalised

least squares (GLS) using the following equation (Saleh et al. 2020):

$$\hat{b} = \left(X^{/}V^{-}X\right)^{-1}X^{/}V^{-}y$$

Where X was the matrix of coefficients of estimable effects of gallinacin gene genotypes, V^{-} = the generalised error variance–covariance matrix, with the variance–covariance matrix of the estimate of 'b' being: $\widehat{\mathbf{Varb}} = (\mathbf{X^{/}V^{-}X})^{-1}$

Results

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 genetic groups studied. The digestion of PCR products in PCR-RFLP tests using the restriction enzymes *HpyCh4IV, AvaI, AluI* and *HinfI* for *GAL2, GAL3, GAL4* and *GAL5* genes indicated that the *GAL2* gene region was monomorphic and *GAL3, GAL4* and *GAL5* genes were polymorphic across R, ½F½R and ½R½F genetic groups. For the gallinacin 2 gene, the PCR-RFLP digestion of DNA samples in parents and F_1 progeny using the *HpyCh4IV* restriction enzyme were monomorphic and included two fragments of 388 and 195 bp (CC genotype). For the gallinacin 3 gene, the PCR-RFLP digestion with *Ava1* produced fragments including a single uncut fragment of 664 bp (TT genotype), two fragments of 443 and 221 bp (CC genotype) and three fragments of 664, 443 and 221 bp (TC genotype). The TT genotype was the common homozygote; *i.e.* the TC genotype was the most common heterozygote and CC genotype was the minor homozygote. For the gallinacin 4 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 gallinacin 5 gene, the PCR-RFLP products of 623, 402 and 133 bp digested by the *HinfI* restriction enzyme represented the major CC homozygote, the CA heterozygote and minor AA homozygote.

Genotypic and allelic frequencies of GAL genes in each genetic group

The allelic and genotypic frequencies of each *GAL* gene per genetic group are shown in Table 1. PCR-RFLP tests showed that the *GAL2* gene was monomorphic and produced a homozygous genotype in the studied genetic groups. The F breed was monomorphic for all *GAL* genes.

For the *GAL3* gene, the frequency of the CC genotype was high in the ½F½R cross chickens. For the TC genotype, the highest frequency was recorded in the R chickens and the lowest frequency in the ½F½R chickens. For the TT genotype, the highest frequency was recorded in the R chickens and the lowest frequency in the ½F½R chickens. The allelic frequency of the *GAL3* gene showed the same trend cited for the genotypic frequency (Table 1).

In the *GAL4* gene, the frequency of the GG genotype was high in the ½F½R chickens. For the AG genotype, the highest frequency was recorded in the R chickens and the lowest frequency in the ½F½R chickens. For the AA genotype, the highest frequency was recorded in the ½R½F chickens and the lowest frequency in the ½F½R chickens. The allelic frequency of the *GAL4* gene showed a similar pattern to that cited for the genotypic frequency, where the highest frequency for the G allele was recorded in the ½F½R chickens (Table 1).

Table 1. Genotypic and allelic frequencies of *GAL3, GAL4* and *GAL5* genes in each genetic group studied.

			Genotype frequency			Allele frequency	
Gene[1]	Breed or genetic group[1]	N	TT	TC	CC	T	C
GAL3	R	24	0.18	0.50	0.32	0.43	0.57
	½R½F	24	0.13	0.33	0.54	0.08	0.92
	½F½R	24	0.00	0.16	0.84	0.21	0.79
			AA	AG	GG	A	G
GAL4	R	24	0.08	0.46	0.46	0.31	0.69
	½R½F	24	0.17	0.33	0.50	0.30	0.70
	½F½R	24	0.00	0.25	0.75	0.125	0.875
			CC	CA	AA	C	A
GAL5	R	24	0.85	0.05	0.10	0.87	0.13
	½R½F	24	0.21	0.79	0.00	0.60	0.40
	½F½R	24	0.17	0.74	0.09	0.54	0.46

[1]*GAL3, GAL4* and *GAL5* = Gallinacin 3, 4 and 5 genes; F = Fayoumi breed was monomorphic; N = number of samples; R = Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R = Fayoumi × Rhode Island Red.

The highest frequency of the AA genotype was in the R and ½F½R chickens. For the CA genotype, the highest frequency was recorded in the ½R½F chickens and the lowest in the R chickens, while for the CC genotype, the highest frequency was recorded in the R chickens and the lowest in the ½F½R crossbred in the *GAL5* gene. The allelic frequency of the *GAL5* gene showed a similar trend to that cited for the genotypic frequency, where the highest frequency for the C allele was recorded in the R chickens (Table 1). These results showed that the *GAL3, GAL4* and *GAL5* genes in the R, ½R½F and ½F½R chickens were polymorphic, because there were three genotypes found with high allelic frequency in each of the fragments obtained.

The effective number of alleles and Hardy-Weinberg equilibrium in each genetic group

The effective number of alleles (N_e) in the studied genetic groups are presented in Table 2, excluding the results of F as monomorphic breed. The highest N_e was obtained in the R breed, followed by the ½R½F cross for an SNP in the *GAL3* gene (T/C_{222}), while the highest N_e was obtained in the R breed, followed by the ½R½F cross, for an SNP in the *GAL4* gene (A/G_{188}). In terms of an SNP in the *GAL5* gene (C/A_{80}), the highest N_e was recorded in the ½F½R cross, followed by the ½R½F cross. The Chi-square values (χ^2) for Hardy-Weinberg equilibrium (HWE) were significant for the *GAL5* gene but not for *GAL3* and *GAL4* genes (Table 2).

The observed (H_o) and expected (H_e) heterozygosities and polymorphism information content (PIC) in each genetic group

The values of H_o and H_e in the genetic groups are shown in Table 3, excluding the monomorphic Fayoumi breed. For the *GAL3* gene, the levels of genetic diversity were intermediate in the studied genetic groups, where the values of H_e were 0.145 in the ½F½R birds and 0.490 in the R breed. Considering the *GAL4* gene, H_o values were higher than H_e values, where H_e ranged from 0.219 in the ½F½R cross to 0.430 in the R breed. For the *GAL5* gene, the values of H_o were higher than the

Table 2. The effective numbers of alleles (N_e) and Chi-square values (χ^2) for Hardy-Weinberg Equilibrium (HWE) characterising *GAL3, GAL4* and *GAL5* genes in each genetic group studied.

Gene	Breed or genetic group[1]	N	Ne	*p*-value	χ^2 value for HWE
GAL3	R	24	1.960	0.709	0.016
	½R½F	24	1.753	0.533	0.389
	½F½R	24	1.170	0.911	0.140
GAL4	R	24	1.753	0.523	0.107
	½R½F	24	1.734	0.392	0.733
	½F½R	24	1.280	0.744	0.408
GAL5	R	24	1.296	0.019	11.256 ***
	½R½F	24	1.917	0.001	10.302 **
	½F½R	24	1.985	0.001	5.512 *

[1]*GAL3, GAL4* and *GAL5* = Gallinacin 3, 4 and 5 genes; Fayoumi breed was monomorphic; N = number of samples; R = Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R = Fayoumi × Rhode Island Red; N_e = effective numbers of alleles; χ^2 = Chi-square value of Hardy-Weinberg Equilibrium (HWE); *P < 0.05, **P < 0.01; ***P < 0.001.

Table 3. The observed (H_o) and expected (H_e) heterozygosities and the polymorphism information content (PIC) for *GAL3, GAL4* and *GAL5* genes in each genetic group studied.

Gene	Breed or genetic group[1]	N	H_o	H_e	PIC
GAL3	R	24	0.476	0.490	0.370
	½R½F	24	0.375	0.430	0.341
	½F½R	24	0.158	0.145	0.136
GAL4	R	24	0.458	0.430	0.336
	½R½F	24	0.615	0.348	0.332
	½F½R	24	0.250	0.219	0.210
GAL5	R	24	0.053	0.229	0.210
	½R½F	24	0.792	0.478	0.365
	½F½R	24	0.739	0.496	0.375

[1]*GAL3, GAL4* and *GAL5* = Gallinacin 3, 4 and 5 genes; F = Fayoumi breed was monomorphic; N = number of samples; R = Rhode Island Red breed; ½R½F = Rhode Island Red×Fayoumi; ½F½R = Fayoumi×Rhode Island Red; H_o = observed heterozygosity; H_e = expected heterozygosity; PIC = polymorphism information content.

values of H_e and H_e and ranged from 0.229 in the R breed to 0.496 in the ½F½R cross. Such low heterozygosity is likely the result of mating between genetically related individuals, and values of observed and expected heterozygosity varied among loci. Evolutionary forces, such as mutation and random genetic drift, may affect loci differently so that it eventually changes the amount of heterozygosity.

Most of the values for PIC given in Table 3 for the R and ½R½F chickens were moderate in *GAL3* and *GAL4* genes and moderate for the ½R½F and ½F½R in the *GAL5* gene (0.25<*PIC*<0.50).

The reduction in heterozygosity due to inbreeding

The F-statistics (F_{IS}, F_{ST} and F_{IT}) presented in Table 4, showed a reduction in heterozygosity due to inbreeding for each gene across all four genetic groups. The estimate of F_{IS} across the *GAL* genes and genetic groups was low (-0.052 ±0.062), which indicated that the F_{IS} values for *GAL2* and *GAL5* genes were very close to zero and had negative magnitude. The highest reduction in heterozygosity due to inbreeding within each population (F_{IS}) was observed in the *GAL3* gene (0.049) and the lowest reduction was observed the in *GAL5* gene (-0.228). The F_{ST} value is the inbreeding coefficient of an individual related to the subpopulation and was calculated from the observed and expected heterozygosity in the subpopulations (Falconer and Mackay 1996). The value of the inbreeding coefficient for the individual relative to the total population (F_{IT}) was high, with an average of 0.071 ±0.057 (Table 4). The results of the four genetic groups could be categorised as a low genetic diversity class.

Table 4. The F-statistics of reduction in heterozygosity (F_{IS}, F_{ST} and F_{IT}) due to inbreeding in each locus across F, R, ½F½R and ½R½F populations.

Locus[1]	F_{IS}	F_{ST}	F_{IT}
GAL2	−0.045	0.033	−0.011
GAL3	0.049	0.165	0.206
GAL4	0.015	0.113	0.126
GAL5	−0.228	0.156	−0.036
Mean ± SE	−0.052 ± 0.062	0.117 ± 0.030	0.071 ± 0.057

[1] F = Fayoumi breed; R = Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R = Fayoumi × Rhode Island Red; F_{IS} = Reduction in heterozygosity due to inbreeding within each population; F_{ST} = Pairwise genetic differentiations among populations; F_{IT} = Reduction in heterozygosity due to inbreeding for each locus; *GAL2, GAL3, GAL4* and *GAL5* = Gallinacin 2, 3, 4 and 5 genes; SE = standard errors.

Molecular associations of genotypes of GAL genes with different studied traits

The generalised least square means (GLMs) given in Table 5 across the four genetic groups showed significant molecular associations of SNP genotypes for the *GAL3* gene (P <0.05) with body weights at 4, 6, 8 and 10 weeks of age. The chicks with the TT genotype at 4, 6, 8 and 10 weeks of age had significantly heavier body weights than the chicks with TC and CC genotypes.

The SNP within the gallinacin 3 gene had insignificant molecular associations with caecal counts of *S. typhimurium* and *E. faecium* and caecal pH, along with IgA, IgG and IgM serum antibody titres across all genetic groups (Table 5).

For all four genetic groups, SNP genotypes in the *GAL4* gene were not significantly associated with the body weights studied (Table 6). The chicks with the AG genotype at two, six, eight and 10 weeks of age had heavier body weights than the chicks with AA and GG genotypes.

The SNP in the gallinacin 4 gene was associated significantly with caecal *S. typhimurium* count (P <0.05), while it

Table 5. Generalised least square means (GLMs) and their standard errors (SE) for body weights and immune traits as affected by SNP genotypes of the *GAL3* gene across F, R, ½F½R and ½R½F chickens.

	Genotypes					
	TT		TC		CC	
Trait[1]	GLM	SE	GLM	SE	GLM	SE
Body weight (BW):						
BW1	70	3.6	68	2.3	66	1.3
BW2	131	8.8	123	5.4	119	3.3
BW4	260[a]	19.8	242[b]	12.2	244[b]	1.1
BW6	487[a]	28.2	447[b]	2.6	433[b]	7.5
BW8	712[a]	35.0	674[b]	21.6	670[b]	13.3
BW10	966[a]	43.5	917[b]	26.8	918[b]	16.6
Immune traits:						
Salmonella typhimurium count (log cfu/g)	2.1	0.52	2.1	0.32	2.1	0.20
Enterococcus faecium count (log cfu/g)	1.9	0.49	2.0	0.30	2.0	0.19
Caecal pH	7.2	0.18	7.1	0.11	7.2	0.07
IgA antibody titre (OD)	1.1	0.22	1.0	0.08	1.0	0.14
IgG antibody titre (OD)	1.1	0.22	1.0	0.08	1.0	0.14
IgM antibody titre (OD)	1.1	0.22	1.0	0.08	1.1	0.14

[1]*GAL3, GAL4* and *GAL5* = Gallinacin 3, 4 and 5 genes; F = Fayoumi breed; R = Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R = Fayoumi × Rhode Island Red; Numbers of records = 96; GLMs = generalised least square means; SE = standard errors; cfu = colony forming unit; OD = optical density; Different letters in the same row indicate significant differences among GLMs at $p < 0.05$.

Table 6. Generalised least square means (GLMs) and their standard errors (SE) for body weights and immune traits as affected by SNPs genotypes of *GAL4* gene across F, R, ½F½R and ½R½F chickens.

	Genotypes					
	AA		AG		GG	
Trait[1]	GLM	SE	GLM	SE	GLM	SE
Body weight (BW):						
BW1	67	1.3	67	2.1	68	5.1
BW2	120	3.2	125	5.2	122	12.4
BW4	242	7.2	249	11.6	269	28.0
BW6	442	10.4	449	16.9	439	40.5
BW8	679	12.8	680	20.7	670	49.7
BW10	923	15.8	931	25.6	916	61.4
Immune traits:						
Salmonella typhimurium count (log cfu/g)	2.2^{a}	0.19	2.1^{ab}	0.31	1.9^{b}	0.75
Enterococcus faecium count (log cfu/g)	1.9	0.18	2.0	0.29	1.9	0.71
Caecal pH	7.2	0.06	7.2	0.10	7.2	0.26
IgA antibody titre (OD)	1.0	0.08	1.1	0.13	1.1	0.32
IgG antibody titre (OD)	1.1	0.08	1.1	0.13	1.1	0.3
IgM antibody titre (OD)	1.0	0.08	1.0	0.13	1.0	0.3

[1]*GAL3, GAL4* and *GAL5* = Gallinacin 3, 4 and 5 genes; F = Fayoumi breed; R = Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R = Fayoumi × Rhode Island Red; Numbers of records = 96; GLMs = generalised least square means; SE = standard errors; cfu = colony forming unit; OD = optical density; Different letters in the same row indicate significant differences among GLMs at $p < 0.05$.

was insignificantly associated with *E. faecium* count, caecal pH and IgA, IgG and IgM serum antibody titres (Table 6). The chicks with the GG genotype had lower caecal *S. typhimurium* counts than those with the AA genotype across all genetic groups.

The SNP within the *GAL5* gene across the four genetic groups showed significant molecular associations of the *GAL5* gene (P <0.05) with body weights at two and eight weeks of age, and the chicks with an AA genotype had significantly heavier body weights than chicks with AC and CC genotypes (Table 7). The SNP in the gallinacin 5 gene was significantly associated with caecal *E. faecium*, but not *S. typhimurium*, counts, caecal pH and IgA, IgG and IgM serum antibody titres (Table 7). For *E. faecium*, the chicks with AC genotype had higher significant bacterial counts than for the AA genotype. For IgA, IgG and IgM serum antibody titres, the differences among the genotypes were non-significant. Accordingly, genotype TT for the *GAL3* gene, genotype AG for the *GAL4* gene and genotype AA for the *GAL5* gene could be regarded as the next parental generation for improving body weights in chickens. The results for immune traits suggested that the genotypes of *GAL4* and *GAL5* genes could be used for marker-assisted selection in breeding programs, where the main goals included the improvement of resistance against *S. typhimurium* in chickens.

Table 7. Generalised least square means (GLMs) and their standard errors (SE) for body weights and immune traits as affected by SNPs genotypes of *GAL5* gene across F, R, ½F½R and ½R½F chickens.

	Genotypes					
	CC		CA		AA	
Trait[1]	GLM	SE	GLM	SE	GLM	SE
Body weight (BW)						
BW1	67	1.4	65	1.6	68	4.8
BW2	124^{ab}	3.6	115^{b}	3.9	139^{a}	12.0
BW4	241	8.0	247	12.8	264	26.9
BW6	444	11.6	442	2.6	449	38.3
BW8	678^{b}	14.2	675^{b}	15.7	731^{a}	47.7
BW10	926	17.7	913	2.6	937	59.6
Immune traits						
Salmonella typhimurium count (log cfu/g)	2.1	0.23	2.0	0.25	2.0	0.77
Enterococcus faecium count (log cfu/g)	1.8^{b}	0.21	2.1^{a}	0.23	1.9^{ab}	0.70
Caecal pH	7.1	0.07	7.2	0.08	7.2	0.25
IgA antibody titre (OD)	1.0	0.09	1.1	0.10	1.1	0.32
IgG antibody titre (OD)	1.0	0.09	1.0	0.10	1.1	0.32
IgM antibody titre (OD)	1.0	0.09	1.0	0.10	1.1	0.32

[1]*GAL3, GAL4* and *GAL5* = Gallinacin 3, 4 and 5 genes; F = Fayoumi breed; R = Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R = Fayoumi × Rhode Island Red; Number of records = 96; GLMs = generalised least square means; SE = standard errors; cfu = colony forming unit; OD = optical density; Different letters in the same row indicate significant differences among GLMs at $p < 0.05$.

Discussion

In this study, the polymorphism impacts of four gallinacin candidate genes on body weight, caecal *S. typhimurium* and *E. faecium* counts and serum antibody titres of F and R breeds and their crosses (½R½F and ½F½R) were investigated. The four gallinacin genes selected for investigation in this study are functional analogues of the mammalian beta-defensins and play an important role in the innate immunity against microbial infections in chickens. Due to the lack of a superoxide ion and myleoperoxidase in avian heterophils, birds rely more upon non-oxidative defence molecules, that include lysozymes, cationic proteins and peptides, such as gallinacins (Hasenstein and Lamont 2007; Zhang et al. 2020). Antimicrobial action is initiated, in principle, by the binding of the peptide to the bacterial membrane through electrostatic interactions (Hasenstein et al. 2006; Zhang et al. 2020). Upon release, antimicrobial peptides such as gallinacins permeate the membrane of bacteria, coinciding with the inhibition of RNA, DNA and protein synthesis (Sugiarto and Yu 2004). Along with their integral role in innate immunity, gallinacins 2 to 5 were of particular interest for analysis, because of their physical proximity in the genome. In the present study, amplified PCR products obtained from the *GAL* genes were similar to those obtained by Hasenstein et al. (2006) in F chickens.

According to the published literature, this is the first study concerning the association of *GAL* genotypes with body weights and immune traits in chickens. The genotypic and allelic frequencies in studied genetic groups indicated differences, which implied that all genetic groups had different polymorphisms at loci within the *GAL3, GAL4* and *GAL5* genes. Manjula et al. (2018) reported that the variabilities in genotypic and allelic frequencies among genetic groups indicated that these genetic differences were in the base population. However, the genotype frequency obtained for *GAL2* SNP did not confirm the Hardy-Weinberg Equilibrium in the F breed, *i.e.* Hardy-Weinberg Equilibrium cannot apply for single genotype. For the *TLR4* gene, Khatab et al. (2017) found that the genotypic frequencies of susceptible birds were 0.4 for BB genotype and 0.6 for AB genotype, while the frequencies in resistant birds of the Hy-Line strain were 0.5 for the BB genotype and 0.5 for the AB genotype in the F breed. The gene frequencies of susceptible birds were 0.7 for the BB genotype and 0.3 for the AB genotype. Ashraf and El-Tarabany (2015) found that the genotypic frequencies of a GG genotype within the *BMPR-1B* gene in F and R chickens were 0.36 and 0.28, respectively.

There are difficulties in comparing the present results of population genetic structures with previous studies, as they were obtained with different genes. However, N_e is an important genetic parameter that was used to show the size of intra-population genetic variation (Tao et al. 2008). Abdalhag et al. (2015) showed that N_e for SNP of *DLEU7, INTS6* and *ATP7B* genes exhibited low or moderate polymorphism with N_e, ranging from 1.033 to 1.773.

Results of the Chi-square testing (χ^2) of the Hardy-Weinberg Equilibrium (HWE) indicated that the differences between the expected and the observed counts for genotypes were limited for *GAL3* and *GAL4* genes. However, the deviations in chi-square values for the *GAL5* gene were significant, which indicated that the R, ½R½F and ½F½R groups were in HWE. These results indicated that allelic frequency was not changed by selection, mutation and hybridisation factors in the studied chicken populations. In the same context, the chi-square values obtained by Abdalhag et al. (2015) indicated that all SNP of *SETDB2, ATP7B, INTS6, DLEU7* and *FOXO1A* genes were in HWE.

The H_o values were higher than H_e values in the R breed and ½F½R cross. This was likely due to the potential population dynamics, selection programme and nature of the sampling process. However, Muhsinin et al. (2017) found that the values of H_o were lower than H_e in different genetic groups. Results for PIC were moderate, and the monomorphic loci in the F breed could be due to the limited sample size and high inbreeding within the population. Tao et al. (2008) stated that PIC analysis is an important genetic parameter that can be used to show the size of intra-population genetic variation. Abdalhag et al. (2015) reported low polymorphism (PIC<0.25) for SNP in *SETDB2, DLEU7* and *ATP7B* genes.

The estimate of F_{IS} across all *GAL* genes and genetic groups was low, which indicated that there was high inbreeding within each population. The low negative F_{IS} values were close to zero for *GAL2* and *GAL5* genes, and were low for *GAL3* and *GAL4* genes; indicating high inbreeding within the populations. There was heterozygous deficiency and/or excess homozygosity. However, such high inbreeding values can be attributed to non-random mating and some loci might be linked to some economic traits. Eltanany et al. (2011) assessed the genetic diversity of three Egyptian chicken breeds (F, Dandarawi and Sinai) and six synthetic lines derived from F and Sinai breeds, and found an F_{IS} value of 0.04. The overall value of F_{ST} was moderate, which showed that there were genetic differentiations among the groups studied. They found that F_{ST} and (F_{IT}) values were 0.07 and 0.11, respectively. The values of inbreeding coefficients of the individual relative to the total population (F_{IT}) were high for *GAL2, GAL3, GAL4* and *GAL5* genes, respectively. These results were confirmed in all SNP genotyped and showed moderate polymorphism across the four genetic groups studied.

The principal novelty of the current research lay in the association of genotypes of *GAL* genes with body weight, caecal *S. typhimurium* count and antibody titres for F and R breeds and their crosses. For the *GAL3* gene, the chicks with a TT genotype had heavier significant body weights than those with TC and CC genotypes at 4, 6, 8 and 10 weeks of age. Supakorn (2016) investigated *ApoB2, TGF-β2, TRAIL* and *IAP1* genes and their associations with growth traits, and reported that only the *ApoB2* gene had a significant association with body weight at eight weeks of age in Thai native chickens. Zhao et al. (2015) examined the *IGFBP-2* gene in Jinghai Yellow chickens and found that chicks with an AA genotype had significantly heavier body weights at hatch and 12 weeks of age, than AB genotype chickens (P <0.05). Kazemi et al. (2018) reported a significant association between the promoter region of the *IL-2* gene and body weight at eight weeks of age in Mazandaran native fowls (P ≤0.05). The differences among the genotypes of the *GAL3* gene and *S. typhimurium* and *E. faecium* counts, caecal pH and serum IgA, IgG and IgM antibody titres were not significant (P =0.27) in the different genetic groups. Hasenstein and Lamont (2007) reported that there was no significant association between the *GAL2* gene sire allele and caecal bacterial loads in progeny and either spleen bacterial load or *S. enteritidis* vaccine antibody response (P =0.10), while Hasenstein et al. (2006) found that the *GAL3* gene was associated significantly with *S. enteritidis* vaccine antibody response in F_1 progeny (P =0.03). Mamutse et al. (2018), when investigating Sentul chickens, showed that a GG genotype in the *TLR4* gene was significantly associated with higher immune traits against *Salmonella spp*. than AG and AA genotypes. However, Muhsinin et al. (2017) found that the TT genotype of the *TGF-β2* gene showed higher resistance to *S. pullorum* compared to TC and CC genotypes in Indonesian chickens (P <0.05). Zhang et al. (2020) reported that SNP 1, 2, 12 and 17 within the *GAL14* gene were significantly associated with sensitivity of *Salmonella spp*., whilst another fifteen SNP in *GAL14* were non-significant, *i.e*. the genotypes TT of SNP1, TT of SNP2, GT of SNP12 and TT and AA of SNP17 were susceptible to *Salmonella spp*. and the genotypes CT and CC of SNP1, AT and AA of SNP2, GG and TT of SNP12 along with AT of SNP17 were resistant.

For the *GAL4* gene, the chicks with AG genotype were significantly heavier in body weight than chicks with AA and GG genotypes at two, six, eight and 10 weeks of age. Molee et al. (2016) reported that the genotypes of the major histocompatibility complex class II gene were significantly associated with body weight at different ages in Leung Hang Khao Thai chickens. Thinh et al. (2019) stated that the GG genotype of the *GH* gene was significantly heavier for body weight than other genotypes. The genotype GG had significantly lower *S. typhimurium* counts than the AA genotype, while there were no significant differences among the genotypes for antibody titres in the studied genetic groups. Kramer et al. (2003) and Malek et al. (2004) showed significant associations between encoding caspase-1, *CD-28, IgL*, and *TRAIL* genes and antibody responses to *S. enteritidis* vaccine in meat-type chickens (outbred broilers lines). Hasenstein et al. (2006) found that the gallinacin 4 gene had no significant associations with either caecal *S. enteritidis* bacterial count (P =0.24) or antibody responses to *S. enteritidis* vaccine (P =0.79).

In chicks from an intercross line, Hasenstein and Lamont (2007) showed that *GAL1, GAL2, GAL4, GAL7, GAL8, GAL9* and *GAL10* had no significant associations with caecal bacterial burden. Zhang et al. (2020) reported that the CT genotype of SNP1, TG genotype of SNP2 and GG genotype of SNP12 of the *GAL4* gene were not associated with susceptibility to *Salmonella spp*. Ardiyana et al. (2020), when studying SenSi-1 Agrinak chickens, stated that the TT genotype of the *iNOS* gene was associated significantly with specific antibody titres against *S. enteritidis*.

With the *GAL5* gene, chicks with AA genotypes had heavier significant body weights than chicks with AC and CC genotypes at two and eight weeks of age. Jin et al. (2018) found that chicks with TT SNP genotype in *Pit-1* gene were significantly heavier for body weight at 10 weeks of age than those chicks with CT and CC genotypes. Manjula et al. (2018) found that the *M_2* SNP of the *POU1F1* gene was associated significantly with body weight at two weeks of age. Genotypes of the *GAL5* gene were not significantly associated with *S. typhimurium* counts or antibody titres in the genetic groups studied. These results are in agreement with Hasenstein et al. (2006) who reported that SNP of the gallinacin 5 gene had no significant associations with antibody response to an *S. enteritidis* vaccine (P =0.11). Tohidi et al. (2013) stated that the *NRAMP1* gene was strongly associated with *S. enteritidis* counts in the caecum (P =0.002) in Malaysian village chickens, and reported that *TGFβ3, TGFβ4* and *TRAIL* were associated with *S. enteritidis* burden in the caecum (P <0.05) in village and Red Jungle Fowl native Malaysian chickens. Muhsinin et al. (2016) reported that the CC genotype of the *NRAMP1* gene in Sentul chickens was significantly lower in S. *pullorum* counts than TC and TT genotypes (P <0.05). Zhang et al. (2020) showed that five SNP in *GAL5* were significantly associated with sensitivity to *Salmonella spp*. Moreover, the AG genotype of SNP2, AA of SNP10, CC of SNP15, CC of SNP16 and TT of SNP17 were found to be sensitive to *Salmonella spp*., while the AA genotype of SNP2, AG and GG of SNP10, TC and TT of SNP15, TC and TT of SNP16 along with TC and CC of SNP17 were found to be resistant to *Salmonella spp*. In Kampung chickens, Ulupi et al. (2013) showed that the AA, AG and GG genotypes of the *TLR4* gene were not significantly associated with IgY antibody titres against *S. enteritidis*. The polymorphisms obtained here for *GAL3, GAL4* and *GAL5* genes and their associations with body weight and resistance to *S. typhimurium* suggested that these genes may be considered as markers for *S. typhimurium* resistance and growth enhancement in chickens.

Conclusions

The TT and AA genotypes of *GAL3* and *GAL5* genes, respectively, had significant associations with body weight. Birds with genotype GG in the *GAL4* gene had a significant reduction in *S. typhimurium* counts, while birds with the CC genotype in the *GAL5* gene had significant reductions in *E. faecium* count.

The gallinacin genes 3, 4 and 5 could be used as candidate genes for marker-assisted selection programs in order to improve body weights and to enhance immune response against *S. typhimurium* in chickens. The molecular associations detected using the SNP markers ($T>C_{222}$, $A>G_{188}$ and $C>A_{80}$ in *GAL3, GAL4* and *GAL5* genes, respectively) may help in identifying effective genotypes for selection programs to improve growth performance and immunity traits in chickens. It can be concluded that PCR-RFLP is a useful tool for screening the gallinacin genes and it can be used efficiently for evaluating genetic variability among different groups of chickens.

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Disclosure statement

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Software and data repository resources

Data are available from the corresponding author upon reasonable request.

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References

Abdalhag, M. A., T. Zhang, Q. C. Fan, X. Q. Zhang, G. X. Zhang, J. Y. Wang, Y. Wei, and Y. J. Wang. 2015. "Single Nucleotide Polymorphisms Associated with Growth Traits in Jinghai Yellow Chickens." *Genetic Molecuar Research* 14: 16169–16177. doi:10.4238/2015.December.8.6.

Ardiyana, M., A. Gunawan, S. Murtini, T. Sartika, and C. Sumantri. 2020. "Polymorphisms and Associations of the NRAMP-1 and iNOS Genes on Newcastle Disease and Salmonella Enteritidis Resistances in SenSi-1 Agrinak Chickens." *Tropical Animal Science Journal* 43: 95–102. doi:10.5398/tasj.2020.43.2.95.

Ashraf, A., and M. S. El-Tarabany. 2015. "Association of Single Nucleotide Polymorphism in Bone Morphogenetic Protein Receptor 1B (BMPR-1B) Gene with Growth Traits in Chicken." *Kafkas Universitesi Veteriner Fakultesi Dergisi Journal* 21: 819–824. Turkey.

Eltanany, M., U. Philipp, S. Weigend, and O. Distl. 2011. "Genetic Diversity of Ten Egyptian Chicken Strains Using 29 Microsatellite Markers." *Animal Genetics* 42 (6): 666–669. doi:10.1111/j.1365-2052.2011.02185.x.

Falconer, D. S., and T. F. C. Mackay. 1996. *Introduction to Quantitative Genetics*. 4th ed. Essex, U.K.: Longman.

Gao, Y., R. Zhang, X. Hu, and N. Li. 2007. "Application of Genomic Technologies to the Improvement of Meat Quality of Farm Animals." *Meat Science* 77 (1): 36–45. doi:10.1016/j.meatsci.2007.03.026.

Groeneveld, E. 2006. *PEST User´s Manual*. FAL, Germany: Institute of Animal Husbandry and Animal Behaviour.

Hasenstein, J. R., and S. J. Lamont. 2007. "Chicken Gallinacin Gene Cluster Associated with Salmonella Response in Advanced Intercross Line." *Avian Diseases* 51 (2): 561–567. doi:10.1637/0005-2086(2007)51[561:CGGCAW]2.0.CO;2.

Hasenstein, J. R., G. Zhang, and S. J. Lamont. 2006. "Analyses of Five Gallinacin Genes and the Salmonella Enterica Serovar Enteritidis Response in Poultry." *Infection and Immunity* 74 (6): 3375–3380. doi:10.1128/IAI.00027-06.

Herigstad, B., M. Hamilton, and J. Heersink. 2001. "How to Optimize the Drop Plate Method for Enumerating Bacteria." *Journal of Microbiological Methods* 44 (2): 121–129. doi:10.1016/S0167-7012(00)00241-4.

Higgs, R., D. J. Lynn, S. Gaines, J. McMahon, J. Tierney, T. James, A. T. Lloyd, G. Mulcahy, and C. O'Farrelly. 2005. "The Synthetic Form of a Novel Chicken β-defensin Identified in Silico Is

Predominantly Active against Intestinal Pathogens." *Immunogenetics* 57 (1–2): 90–98. doi:10.1007/s00251-005-0777-3.

Jin, S., T. He, L. Yang, Y. Tong, X. Chen, and Z. Geng. 2018. "Association of Polymorphisms in Pit-1 Gene with Growth and Feed Efficiency in Meat-type Chickens." *Asian-Australasian Journal of Animal Sciences* 31 (11): 1685–1690. doi:10.5713/ajas.18.0173.

Kaiser, M. G., and S. J. Lamont. 2001. "Genetic Line Differences in Survival and Pathogen Load in Young Layer Chicks after Salmonella Enterica Serovar Enteritidis Exposure." *Poultry Science* 80 (8): 1105–1108. doi:10.1093/ps/80.8.1105.

Kalinowski, S. T., M. L. Taper, and T. C. Marshall. 2007. "Revising How the Computer Program CERVUS Accommodates Genotyping Error Increases Success in Paternity Assignment." *Molecular Ecology* 16 (5): 1099–1106. doi:10.1111/j.1365-294X.2007.03089.x.

Kazemi, H., M. Najafi, E. Ghasemian, G. Rahimi-Mianji, and Z. A. Pirsaraei. 2018. "Polymorphism Detection of Promoter Region of IFN-γ and IL-2 and Their Association with Productive Traits in Mazandaran Native Breeder Fowls." *Journal of Genetics* 97: 843–851. doi:10.1007/s12041-018-0981-1.

Khatab, S. A., S. A. Hemeda, A. F. El-Nahas, W. S. Abd, and E. Naby. 2017. "Polymorphisms of TLR4 Gene and Its Association with Genetic Resistance to Salmonella Enteritidis Infection in Fayoumi Breed and Hyline Strain in Egypt." *Alexandria Journal for Veterinary Sciences* 55 (2): 1–9. doi:10.5455/ajvs.273717.

Kramer, J., M. Malek, and S. J. Lamont. 2003. "Association of Twelve Candidate Gene Polymorphisms and Response to Challenge with Salmonella Enteritidis in Poultry." *Animal Genetics* 34 (5): 339–348. doi:10.1046/j.1365-2052.2003.01027.x.

Kulibaba, R. A., and A. P. Podstreshnyi. 2012. "Prolactin and Growth Hormone Gene Polymorphisms in Chicken Lines of Ukrainian Selection." *Cytology and Genetics* 46 (6): 390–395. doi:10.3103/S0095452712060060.

Liu, L., Z. Cui, Q. Xiao, H. Zhang, X. Zhao, Y. Wang, H. Yin, D. Li, and Q. Zhu. 2018. "Polymorphisms in the Chicken Growth Differentiation Factor 9 Gene Associated with Reproductive Traits." *BioMed Research International* 2018: 1-1. online. doi:10.1155/2018/4963834.

Malek, M., J. R. Hasenstein, and S. J. Lamont. 2004. "Analysis of Chicken TLR4, CD28, MIF, MD-2, and LITAF Genes in a Salmonella Enteritidis Resource Population." *Poultry Science* 83 (4): 544–549. doi:10.1093/ps/83.4.544.

Mamutse, J., A. Gunawan, C. Sumantri, S. Murtini, and T. Sartika. 2018. "'Association of the Toll-like Receptor 4 (TLR4) and Myxovirus (Mx) Genes with Resistance to Salmonella and Newcastle Disease in Selected Sentul Chickens." *Poultry Science* 17: 591–599. doi:10.3923/ijps.2018.591.599.

Manjula, P., N. Choi, D. Seo, and J. H. Lee. 2018. ""POU Class 1 Homeobox 1 Gene Polymorphisms Associated with Growth Traits in Korean Native Chicken"." *Asian-Australasian Journal of Animal Sciences* 31 (5): 643–649. doi:10.5713/ajas.17.0354.

Molee, A., K. Kongroi, P. Kuadsantia, C. Poompramun, and B. Likitdecharote. 2016. "Association between Single Nucleotide Polymorphisms of the Major Histocompatibility Complex Class II Gene and Newcastle Disease Virus Titre and Body Weight in Leung Hang Khao Chickens." *Asian-Australasian Journal of Animal Sciences* 29 (1): 29–35. doi:10.5713/ajas.15.0029.

Muhsinin, M., N. Ulupi, A. Gunawan, I. Wibawan, and C. Sumantri. 2016. "Association of NRAMP1 Polymorphisms with Immune Traits in Indonesian Native Chickens." *International Journal of Poultry Science* 15: 401–406.

Muhsinin, M., N. Ulupi, A. Gunawan, I. W. T. Wibawan, and C. Sumantri. 2017. "G. 640T> C Polymorphism of the TGF-β2 Gene Is Associated with Salmonella Pullorum Resistance in Indonesian Chickens." *Animal Production* 19 (2): 81–92. doi:10.20884/1.jap.2017.19.2.597.

Peakall, R., and P. E. Smouse. 2012. "GenAlEx 6.5: Genetic Analysis in Excel. Population Genetic Software for Teaching and Research an Update." *Bioinformatics* 28 (19): 2537–2539. doi:10.1093/bioinformatics/bts460.

Raymond, M. 1995. "GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism." *Journal of Heredity* 86: 248–249. doi:10.1093/oxfordjournals.jhered.a111573.

Saleh, M. S. 2019. "Using Bioinformatics SNP to Improve Immune Genetic Response against Some Pathogens in Poultry." M Sc. thesis in cooperation between Faculty of Agriculture at Moshtohor, Benha University and TEMPUS European Union program, Egypt.

Saleh, M. S., M. M. Iraqi, M. H. Khalil, and A. Camarda. 2020. "Crossbreeding Analyses and Polymorphic Associations of Gallinacin Genes with Growth Traits in Chickens." *Livestock Science* 240 (2020): 104–118. doi:10.1016/j.livsci.2020.104118.

Saxena, V. K., and G. Kolluri. 2018. "Selection Methods in Poultry Breeding: From Genetics to Genomics. Application of Genetics and Genomics in Poultry Science." Accessed 4 January 2020. doi:10.5772/intechopen.77966

Sugiarto, H., and P. Yu. 2004. "Avian Antimicrobial Peptides: The Defense Role of β-defensins." *Biochemical and Biophysical Research Communications* 323 (3): 721–727. doi:10.1016/j.bbrc.2004.08.162.

Supakorn, C. 2016. "Genetic Polymorphism of ApoB2, TGF-β2, TRAIL and IAP1 Genes and Their Associations with Growth Traits in Thai Native Chicken." *Aisian Journal of Poultry Science* 10: 141–146.

Tao, Y., G. H. Li, Y. P. Hu, M. D. Mekki, K. W. Chen, and J. Y. Wang. 2008. "Genetic Effects of MC4R and POU1F1 Gene on the Growth Performance in Jinghai Yellow Chicken." *Yi Chuan* 30 (7): 900–906. doi:10.3724/SP.J.1005.2008.00900.

Thinh, N. H., H. A. Tuan, N. T. Vinh, B. H. Doan, N. T. P. Giang, F. Frédéric, M. Nassim, N. V. Linh, and P. K. Dang. 2019. "Association of Single Nucleotide Polymorphisms in the Insulin and Growth Hormone Gene with Growth Traits of Mia Chicken." *Indian Journal of Animal Research* 54 (6): 661–666. doi:10.18805/ijar.B-955.

Tohidi, R., I. B. Idris, J. M. Panandam, and M. H. Bejo. 2013. "The Effects of Polymorphisms in 7 Candidate Genes on Resistance to Salmonella Enteritidis in Native Chickens." *Poultry Science* 92 (4): 900–909. doi:10.3382/ps.2012-02797.

Ulupi, N., C. Sumantri, and I. Wibawan. 2013. "Association of TLR4 Gene Genotype and Resistance against Salmonella Enteritidis Natural Infection in Kampung Chicken." *International Journal of Poultry Science* 13: 467–472. doi: 10.3923/ijps.2014.467.472.

Xiao, Y., A. L. Hughes, J. Ando, Y. Matsuda, J. Cheng, D. Skinner-Noble, and G. Zhang. 2004. "A Genome-wide Screen Identifies A Single β-defensin Gene Cluster in the Chicken: Implications for the Origin and Evolution of Mammalian Defensins." *BMC Genomics* 5 (1): 56. doi:10.1186/1471-2164-5-56.

Zelver, N., M. Hamilton, B. Pitts, D. Goeres, D. Walker, P. Sturman, and J. Heersink. 1999. "Measuring Antimicrobial Effects on Biofilm Bacteria: from Laboratory to Field."*Methods in Enzymology* 310: 608–628.

Zhang, L., M. Huang, Y. Li, D. Chen, and X. Shi. 2020. "Association of Three Beta-defensin Gene (Avbd4, AvBD5, AvBD14) Polymorphisms with Carrier-state Susceptibility to Salmonella in Chickens." *British Poultry Science*. May. 1–9. doi:10.1080/00071668.2020.1752913.

Zhao, X., M. Li, S. Xu, and G. Liu. 2015. "Single Nucleotide Polymorphisms in IGFBP-2 Gene and Their Associations with Body Weight Traits on Jinghai Yellow Chicken." *Brazilian Journal of Poultry Science* 17: 497–502. doi:10.1590/1516-635X1704497-502.