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Modelling effects of phytobiotic administration on coherent responses to *Salmonella* infection in laying hens

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ABSTRACT
Practice of layer poultry farming and commercial egg production relies on the optimal use and improvement of the welfare and genetically determined functional abilities of laying hens, their efficient intake of feed and its components, adaptation to housing conditions and resistance to infectious diseases including salmonellosis. Previous studies were focussed on relationships of chicken performance and resistance with the expression profiles of individual genes involved in metabolic processes and immune system, or with genetic markers that can be closely associated with these processes in chickens. In this study, mathematical models of coherent changes in laying hens were developed for the expression of eight genes involved in immunity and metabolism, on the one hand, and biochemical and immunological blood parameters, on the other hand, in response to *Salmonella* infection and administration of a phytobiotic Intebio. The proposed modelling approach can be a further basis for an in-depth research of the relationship between the gene expression, functional state and welfare of poultry, impact of pathogenic microorganisms and use of immunomodulatory drugs.

HIGHLIGHTS
- Improved egg production and resistance rely on hens' potential in gene expression and metabolism.
- We developed mathematical models for coherent responses of hens' gene expression and blood indices to *Salmonella* infection and phytobiotic intake.
- This approach can be used for further studying relationship between gene expression, functional state, impact of pathogens and antimicrobial drugs.

Introduction

Salmonellosis remains a serious problem both in poultry industry and in public health care sector (Mughini-Gras et al. 2014), while the development of new approaches and veterinary drugs to combat the *Salmonella* bacteria continues to be relevant (Akbari et al. 2008; Yang et al. 2015).

In chickens (*Gallus gallus*), welfare and overall functional status of the organism can be described quite complicatedly and depends both on the individual genetic characteristics and genetic potential, and on the influence of environmental factors experienced by birds in actual growing, housing and feeding conditions. One of the important components of poultry welfare and functional status are the expression levels of certain genes and the state of metabolism, which, in turn, can be estimated via blood biochemical and immunological parameters (e.g. Avzalov 2003; Kletikova and Pronin 2014; Topuriya 2017). Herewith, in response to various environmental factors affecting the bird's

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Table 1. Correlation coefficients (r)* between gene expression and blood biochemical/immunological parameters.

Blood parameters	Genes							
	AvBD10	IL6	IL8L1	SLC5A1	CA2	CALB1	RARRES1	BPIFB3
Total protein, g/L	−0.173	0.102	−0.225	−0.369	0.183	0.187	−0.175	−0.182
Albumin, g/L	0.228	0.386	0.275	−0.039	−0.135	−0.292	0.068	0.005
Globulins, g/L	−0.242	−0.079	−0.374	−0.333	0.378	0.448	−0.263	−0.128
Urea, mmol/L	**−0.531**	**−0.720**	**−0.551**	**−0.574**	0.365	0.292	**−0.581**	−0.242
Urea nitrogen, mmol/L	**−0.528**	**−0.690**	**−0.547**	**−0.586**	0.398	0.412	**−0.588**	−0.264
Creatinine, μmol/L	−0.252	−0.332	−0.286	−0.112	0.340	0.205	−0.216	0.055
Alanine aminotransferase, IU/L	0.107	−0.314	0.051	0.030	−0.101	−0.113	0.032	**−0.573**
Aspartate aminotransferase, IU/L	−0.155	−0.326	0.494	−0.029	−0.126	−0.473	−0.116	0.294
Alkaline phosphatase, IU/L	0.222	0.390	0.010	0.305	−0.214	−0.181	0.330	0.156
Alpha amylase, IU/L	−0.002	0.239	**0.508**	**0.606**	−0.361	−0.347	**0.505**	0.361
Glucose, mmol/L	0.067	0.333	**0.752**	0.483	**−0.552**	−0.443	0.421	**0.552**
Total cholesterol, mmol/L	0.421	0.294	−0.490	−0.296	0.241	0.266	−0.068	−0.395
Calcium, mmol/L	0.107	0.096	**−0.560**	**−0.566**	0.291	0.380	−0.326	−0.497
Phosphorus, mmol/L	0.396	0.218	−0.356	−0.324	−0.180	−0.043	−0.051	−0.357
Bilirubin, μmol/L	0.348	0.024	−0.211	0.066	−0.145	−0.148	0.194	**−0.509**
Uric acid, μmol/L	−0.017	0.390	0.044	−0.181	−0.161	−0.111	−0.048	0.226
Lysozyme activity, %	0.363	0.122	−0.453	−0.209	**0.521**	0.449	−0.185	−0.377
Bactericidal activity, %	0.216	0.139	0.160	0.158	−0.305	−0.068	0.175	−0.329
Beta-lysine activity, %	0.131	−0.075	0.222	0.037	−0.358	−0.205	−0.012	**−0.515**
Activity of immunoglobulin IgA	−0.136	−0.296	**−0.647**	**−0.554**	0.176	0.261	−0.384	−0.141
IgM	0.423	0.013	−0.453	−0.424	0.349	0.287	−0.257	**−0.670**
IgG1	0.420	0.392	0.190	0.057	−0.162	−0.207	0.151	0.067
IgG2	0.220	0.408	**0.850**	**0.605**	−0.316	**−0.506**	0.467	**0.623**

*Pairwise correlation coefficients with $|r| > 0.5$ are given in bold.
IgM: immunoglobulin IgM; IgG1: immunoglobulin IgG1; IgG2: immunoglobulin IgG2.

organism, certain genes involved in a number of metabolic pathways can be activated or suppressed.

As an example of earlier searches for association of the functioning of single genes with economically important traits in poultry, one can mention previous genetic studies on activity of serum enzymes. For instance, tissue-nonspecific alkaline phosphatase controlled by the *ALPL* gene has long been known to be polymorphic and have two allele types, F (fast) and S (slow), found in chickens of different breeds. It was observed on electrophoregrammes that the activity of the dominant fast type was 4–5 times greater than that of the recessive slow type (e.g. Bondarenko and Kutnyuk 1975; Bondarenko 1976). Under the influence of stress factors, an increase in alkaline phosphatase activity was shown in laying hens (Kletikova and Pronin 2014). At the population level, applicability of polymorphic blood groups at 6 genetic loci (Bondarenko and Podstreshny 1996) and erythrocyte antigens, when defining population genetic information profiles by the level of entropy (Katerinich et al. 2014), was also demonstrated.

Recently, we showed the immunomodulating properties of a novel feed additive Intebio based on essential oils and its positive impact on the welfare of laying hens (Dubrovin et al. 2019). When Intebio was added to the ration of laying hens, a more effective launch of immune response, increased resistance, and improved egg weight were observed. Immune response in birds challenged with *Salmonella*

Enteritidis (SE) and fed the additive was more pronounced, and a lowering of egg production curve with age proceeded more smoothly.

In the present work, an attempt was made to develop mathematical models in order to evaluate the effects of intake of phytobiotic Intebio and SE infection in laying hens on the coherent regulation of genes and metabolic responses that seem to be instrumental in maintaining functional state and welfare of poultry.

Materials and methods

Experimental design

To assess the effects of administration of essential oils-based phytobiotic Intebio and SE challenge on functional state and welfare of laying hens, a correlation analysis was performed between the expression data of eight genes and 23 blood biochemical/immunological parameters (Table 1) obtained for 24 females of the Lohmann White cross in a study as previously described by Dubrovin et al. (2019). In short, the experiment included chickens over 300 days old that were subjected to infection with a SE epizootic strain and Intebio supplementation in the diet. The phytobiotic intake experiment was started at 346 days of age of layer hens, and at 367 days of age they were inoculated with the SE strain. At 1-day post infection, tissue samples of the hens' caecum were taken for the further laboratory analysis and used for gene expression examination. Total RNA from the caecal samples was isolated using

Table 2. Accuracy of calculating the relationship of gene expression with biochemical/immunological blood parameters based on their multiple correlation.

Genes	Correlated blood parameters	Calculation accuracy (R^2)	Relationship closeness (R)
AvBD10	*U, NU*	0.502	0.709
IL6	*U, NU*	0.538	0.733
IL8L1	*U, NU, Am, Gl, Ca, IgA, IgG2*	0.855	0.925
SLC5A1	*U, NU, Am, Ca, IgA, IgG2*	0.556	0.746
CA2	*Gl, La*	0.518	0.720
CALB1	*IgG2*	0.290*	0.538*
RARRES1	*U, NU, Am*	0.688	0.829
BPIFB3	*Alt, Gl, Br, Bla, IgM, IgG2*	0.758	0.870

U: urea; *UN*: urea nitrogen; *Alt*: alanine aminotransferase; *Am*: alpha amylase; *Gl*: glucose; *Ca*: calcium; *Br*: bilirubin; *La*: lyso-zyme activity; *Bla*: beta-lysine activity; *IgA*: immunoglobulin IgA; *IgM*: immunoglobulin IgM; *IgG2*: immunoglobulin IgG2.
*Single pairwise correlation.

the Aurum^{TM} Total RNA Mini Kit (BioRad, USA), and reverse transcription reaction was performed to produce cDNAs from RNA templates by employing the iScript^{TM} Reverse Transcription Supermix Kit (Bio-Rad) according to the manufacturer's instructions.

It was hereby assumed that taking into account the aggregated data for the entire experimental sampling would quite noticeably facilitate conformity of the cor-relation results. Thus, provision of a much greater vari-ability of the analysed data was expected, expanding thereby feasibilities of mathematical description of probable variants for coherent changes in gene expression and blood biochemical/immunological indi-ces, which would reflect the involvement of certain metabolic pathways in maintaining the functional state and welfare of the bird's body.

Gene expression analysis

Differential expression was evaluated for the following genes involved in immunity, transport and metabolism (Table 1): *AvBD10*, avian β-defensin 10 (antimicrobial peptide); *IL6*, interleukin 6 (response to inflammation and infection); *IL8L1*, interleukin 8-like 1 (inflammatory activity); *SLC5A1*, solute carrier family 5 member 1 (sodium/glucose transport); *CA2*, carbonic anhydrase 2 (HCO_3^- and CO_2 transport, nitrogen metabolism and eggshell formation); *CALB1*, calbindin 1 (calcium ion transport); *RARRES1*, retinoic acid receptor responder 1 (negative regulation of endopeptidase activity and eggshell matrix protein); and *BPIFB3*, BPI fold contain-ing family B member 3 (innate immune response and eggshell matrix protein). Prior to assessing correlation coefficients (*r*), expression values of the eight studied genes were normalised in between the samples rela-tive to beta actin used as a household gene.

Results and discussion

The results of the correlation analysis are presented in Table 1. For the subsequent mathematical analysis, pairs of parameters were chosen that showed a correlation of at least 0.5 (i.e. at $|r| > 0.5$; Table 1). Graphic dependen-cies for the corresponding mathematical functions reflecting pairwise coherent changes in gene expression and blood indices are given in the Supplemental Material, Figures SM1–SM8. For example, for the *IL8L1* gene expression, the following biochemical/immuno-logical parameters meet this criterion (Table 1): the levels of urea (−0.551), urea nitrogen (−0.547), alpha amylase (0.508), glucose (0.752), calcium (−0.560), and immuno-globulins IgA (−0.647) and IgG2 (0.850). A more detailed mathematical analysis of the corresponding correlations is depicted in the graphs in the Supplemental Material, Figure SM1 (A–G), each of which was approximated by the most accurate mathematical dependence.

To estimate the multiple correlation of these blood indices with *IL8L1*, their values were approximated by an equation based on the product of power functions:

$$IL8L1 = 1.746 U^{0.05} \cdot NU^{-0.076} \cdot Am^{-0.204} \cdot Gl^{0.51} \cdot Ca^{-0.129}$$
$$\cdot IgA^{-0.241} \cdot IgG2^{0.095},$$

$$(1)$$

$$R^2 = 0.855 \text{ (Table 2)},$$

where *U*, *NU*, *Gl*, and *Ca* are concentrations of urea, urea nitrogen, glucose and calcium, respectively; and *Am*, *IgA*, and *IgG2* are activities of alpha amylase and immunoglo-bulins IgA and IgG2 in the blood of laying hens.

The accuracy of approximation by Equation (1) is quite high ($R^2 = 0.855$) and exceeds the maximum cal-culation accuracy ($R^2 = 0.785$) for a single pairwise dependence (see the Supplemental Material, Figure SM1G), ensuring the relationship closeness at $R = 0.925$ (Table 2). As a result, this calculation formula can be taken for mathematical description of corre-lated changes in the levels of *IL8L1* gene expression and blood biochemical/immunological parameters, which to a certain extent reflects the contribution of these variables to the bird's functional state.

Similar mathematical processing of the data was carried out for other genes taken into account

recalculation options for both pairwise combinations and multiple correlation relations.

For the *AvBD10* gene expression (see the Supplemental Material, Figure SM2), the highest correlations with the content of urea ($r = -0.531$) and urea nitrogen ($r = -0.528$) were found (Table 1). Taking into consideration the nature of their dispersion and the evaluation of single pairwise relationships of the *AvBD10* gene expression with these two blood indices, a multiple correlation dependence was obtained as follows:

$$AvBD10 = 2.433U^{-0.0009} \cdot NU^{-0.083}, \quad (2)$$
$$R^2 = 0.224$$

Accuracy of the above formula (2) is rather low and clearly inferior to single pairwise calculations for the parameters U and NU; however, it gives an idea of the degree of contribution of each of the indicators to the overall dependence. The exponent at U is quite small and its contribution to the combined multiple correlation dependence is only 1%. Thus, one can suggest that the content of nitrogen urea is a more significant blood index for the coherent *AvBD10* gene expression. Nevertheless, we tried to improve the accuracy of this calculation by selecting other parametric equations, with the result that the following formula provided a greater accuracy:

$$AvBD10 = 2.44 + 1.485U - 3.073NU + 0.545U$$
$$\cdot NU - 0.305U^2 + 0.151NU^2, \quad (3)$$
$$R^2 = 0.502 \text{ (Table 2)}.$$

The closeness level of the multiple relation of *AvBD10* gene expression with the content of urea and urea nitrogen in the blood was $R = 0.709$ (Table 2), enabling the calculation formula (3) be adopted for interpreting consistent changes in these components of the bird's functional state.

Similar to the *AvBD10* gene, the highest correlations of the *IL6* gene expression (see the Supplemental Material, Figure SM3) were also determined with the content of urea ($r = -0.720$) and urea nitrogen ($r = -0.690$). The analysis of multiple correlation performed similarly to the calculation of *AvBD10* gene expression made it possible to compare two kinds of calculation formulas:

$$IL6 = 2.735U^{-0.191} \cdot NU^{0.077}, \quad (4)$$
$$R^2 = 0.449,$$

and

$$IL6 = 2.541 + 0.35U - 0.88NU + 0.12U \cdot NU - 0.082U^2$$
$$+ 0.095NU^2, \quad (5)$$
$$R^2 = 0.538 \text{ (Table 2)}.$$

Among these equations, formula (5) turned out to be more accurate (with the relation closeness of the parameters being $R = 0.733$; Table 2), as a result of which this formula was adopted to describe the coherent changes of these correlated indices of the functional status of laying hens.

Expression of the *SLC5A1* gene (see the Supplemental Material, Figure SM4) was most associated with the following blood biochemical/immunological parameters in the studied birds (Table 1): content of urea ($r = -0.574$), urea nitrogen ($r = -0.586$) and calcium ($r = -0.566$), and activities of alpha amylase ($r = 0.606$) and immunoglobulins IgA ($r = -0.554$) and IgG2 ($r = -0.605$). The multiple regression model enabled an increased accuracy in calculating the *SLC5A1* gene expression ($R = 0.746$; Table 2) after approximating the experimental data with the following dependence:

$$SLC5A1 = 1.512U^{-0.095} \cdot NU^{0.035} \cdot Am^{-0.043} \cdot Ca^{-0.155}$$
$$\cdot IgA^{0.052} \cdot IgG2^{0.045}, \quad (6)$$
$$R^2 = 0.556 \text{ (Table 2)}.$$

Correlation analysis of *CA2* gene expression (see the Supplemental Material, Figure SM5) revealed its relationship with glucose content ($r = -0.552$) and lysozyme activity ($r = 0.521$; Table 1). The analysis of the closeness of the multiple relationships between these indicators resulted in the following calculation formula (with the accuracy at $R = 0.720$; Table 2):

$$CA2 = 3.079 - 0.191Gl - 0.084La - 0.015Gl \cdot La$$
$$+ 0.014Gl^2 + 0.012La^2, \quad (7)$$
$$R^2 = 0.518 \text{ (Table 2)},$$

where *La* is the level of lysozyme activity.

Analysis of *CALB1* gene expression (see the Supplemental Material, Figure SM6) revealed its relatively strong correlation with only one blood immunological indicator, activity of immunoglobulin IgG2 ($r = -0.506$; Table 1). The resulting calculation formula is not very accurate ($R = 0.538$; Table 2), and its use is more likely to be appropriate only for an approximate assessment of correlated changes in the expression of this gene and activity of immunoglobulin IgG2:

$$CALB1 = 0.0007IgG2^2 - 0.0219IgG2 + 1.0308, \quad (8)$$
$$R^2 = 0.290 \text{ (Table 2)}.$$

Correlation analysis of the dependence of *RARRES1* gene expression on blood biochemical parameters (see the Supplemental Material, Figure SM7) made it

possible to detect its highest relationships with the levels of urea ($r = -0.581$), urea nitrogen ($r = -0.588$) and alpha amylase ($r = 0.505$; Table 1). The multiple correlation between these indicators, as described by the product of power functions, was represented by the following formula, which turned out to be the most accurate, ensuring the closeness of the relationship between these values at $R = 0.829$ (Table 2):

$$RARRESI = 3.127 - 0.187U - 0.849NU - 0.003Am$$
$$+ 0.099U \cdot NU + 0.000075U \cdot Am +$$
$$+ 0.00086NU \cdot Am - 0.0075U^2 + 0.0174NU^2 \quad (9)$$
$$+ 0.000001Am^2,$$
$$R^2 = 0.688 \text{ (Table 2)}.$$

Relatively high correlations of the *BPIFB3* gene (see the Supplemental Material, Figure SM8) were found with the following blood biochemical/immunological indices (Table 1): content of alanine aminotransferase ($r = -0.573$), glucose ($r = 0.552$) and bilirubin ($r = -0.509$), and activities of beta-lysine (-0.515) and immunoglobulins IgM ($r = -0.670$) and IgG2 ($r = 0.623$). The analysis of the closeness of the multiple relationships between these parameters allowed us to derive the following calculation formula:

$$BPIFB3 = 14.924Alt^{-0.04} \cdot Gl^{0.592} \cdot Br^{0.024} \cdot IgM^{-0.028}$$
$$\cdot IgG2^{0.079} \cdot Bla^{-0.835},$$
$$(10)$$
$$R^2 = 0.758 \text{ (Table 2)}$$

where *Alt* is alanine aminotransferase activity; *Br* is bilirubin content; *IgM* is activity of immunoglobulin IgM and *Bla* is beta-lysine activity.

Formula (10) in its accuracy exceeds the accuracy of calculations using single pairwise relationships, ensuring the closeness of the multiple relationship at $R = 0.870$ (Table 2), and can be used as a basis for characterising the coherent changes in the *BPIFB3* gene expression and blood biochemical/immunological indices correlated with it.

The practical use of the regression equations allows for a fairly accurate determination of the correlation of consistent changes in gene expression and various blood biochemical/immunological parameters ($R = 0.709$–0.937), except for the *CALB1* gene ($R = 0.538$), for which only one pairwise correlation with a single blood immunological indicator was established (Table 2). Perhaps, in the future an additional study should be done on peculiarities of this gene expression relative to changes in certain components of the bird's functional status.

No essential correlation was found for alkaline phosphatase and few other blood indices (Table 1). The most informative blood biochemical/immunological parameters were the content of urea, urea nitrogen and glucose, and activity of immunoglobulin IgG2 that most strongly correlated with coherent changes in the expression of eight genes studied in this experiment. This can serve as a basis for further deeper studies on biologically meaningful relationship of blood biochemical/immunological characteristics with genetic parameters, which would bring to a better understanding of various components that ensure the functional state and welfare of the bird's body (Cogburn et al. 2003, 2007; Avzalov 2003; Topuriya 2017).

Sanitary and epidemiological aspects are of great importance for the dynamic development of poultry industry and an increase in the consumption of poultry meat and eggs, since the safety of poultry products is increasingly coming to the fore. In this study, we have proposed and examined one of the modelling-based analytical approaches that can be used in practice and for further research in this area.

The main objectives and advantages of mathematical modelling relevant to various biological processes can be considered as (i) a more evident understanding of the fundamental functioning mechanisms of these processes, and (ii) a solution of certain applied problems more related to the purpose of a specific study.

As for mathematical modelling of gene expression, the fundamental grounds of this approach have been well studied and, as reviewed by some authors (Yīlmaz et al. 2005; Ay and Arnosti 2011; Robert 2019), can be represented by the following major models: differential, stochastic (probabilistic), kinetic and thermodynamic ones. The main benefits of such options for the mathematical structuring of gene expression are transition from a qualitative description to a quantitative estimation and understanding of the laws of gene expression functioning.

Nevertheless, the question of the practical determination of the expression level of any particular genes examined in a particular study often remains open, given the limited possibility of carrying out the necessary measurements of all parameters that affect the course of gene expression process, suggesting other ways for rapid analysis. In this regard, the mathematical dependencies presented by us here would be indispensable, since they allow to quickly and accurately calculate all the necessary factors of gene expression by using the minimum set of initial parameters that can be obtained in any correspondent biological laboratory.

Conclusions

Overall, results of the mathematical modelling can be of practical interest as well as a prerequisite for a

more thorough study on correlation between gene expression and blood biochemical/immunological parameters of the examined biological organisms, especially under conditions of infection with *Salmonella* and other pathogenic microorganisms, as well as testing veterinary drugs that can improve poultry resistance, performance and welfare.

Ethical approval

Animal care and experimental protocol were set up according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, ETS No 123 and was authorised by the relevant national authority. The ethical policies have been adhered to and the appropriate ethical review committee approval has been received.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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